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**An investigation on the effects of bovine
papillomavirus type 4 E5 protein on major
histocompatibility complex class I**

Barbara Marchetti

August 2006

**This thesis is submitted to the University of Glasgow in
accordance with the requirements for the degree of
Doctor of Philosophy in the Faculty of Veterinary Medicine**

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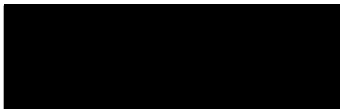
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Declaration:

‘Unless stated otherwise all work contained within this thesis has been completed personally’



Barbara Marchetti

August 2006

Abstract

Papillomaviruses (PV) are small DNA tumour viruses that infect mucosal and cutaneous epithelium where they induce benign hyperproliferative lesions. In the great majority of cases, papillomavirus infections are usually cleared, after several months by a cell mediated immune response directed against viral antigens (Frazer and Tindle 1996). Occasionally, however, the lesions do not regress and can progress to cancer under appropriate environmental conditions. Failure of virus clearance is attributed to a poor immunological response and persistent viral infection poses a greater risk of neoplastic progression.

Together with E6 and E7, E5 is a transforming protein of PV. While E6 and E7 are the main oncoproteins of mucosal human papillomavirus (HPVs) (Munger *et al.* 2001; Mantovani and Banks 2001), E5 is the major oncoprotein of bovine papillomaviruses (BPVs), particularly of BPV-1 (DiMaio and Mattoon 2001). The E5 protein is a small hydrophobic peptide (from 83 amino acid residues in HPV-16 to 42 residues in BPV-4) which is expressed during the early stages of infection in the deep layers of the infected epithelium and its expression is extinguished as the lesion progresses (Burnett *et al.* 1992; Anderson *et al.* 1997; Chang *et al.* 2001).

In agreement with its hydrophobic nature, E5 is localized in the endomembrane compartments of the endoplasmic reticulum (ER) and Golgi apparatus (GA) of the host cell (Burkhardt *et al.* 1989; Pennie *et al.* 1993). The function of E5 *in vivo* is not known, but *in vitro* cell transformation is brought about by the activation of several kinases, from growth factor receptors to cyclins-cdks (Morgan and Campo 2000). E5 interacts physically with the cellular protein 16k ductin/subunit c, a component of the gap junction and of the V0 sector of the vacuolar H⁺-ATPase (Goldstein *et al.* 1991; Conrad *et al.* 1993; Finbow *et al.* 1995; Faccini *et al.* 1996). This physical interaction

has been deemed responsible for the down-regulation of gap junction communication (Oelze *et al.* 1995; Faccini *et al.* 1996; Ashrafi *et al.* 2000) and for the lack of acidification of endosomes and GA and the consequent impaired functions of these organelles (Straight *et al.* 1995; Schapiro *et al.* 2000). Previous studies have shown that transformed cells expressing BPV-1 E5 or BPV-4 E5 retain major histocompatibility class I (MHC I) molecules in the Golgi apparatus and cause the inhibition of transport of the complex to the cell surface (Marchetti *et al.* 2002; Ashrafi *et al.* 2002).

This effect can be ascribed to a failure of acidification of the Golgi apparatus, as similar effects are observed in control cells treated with the ionophore monensin.

In addition, the total amount of both MHC I protein and mRNA is reduced in E5-transformed cells (Ashrafi *et al.* 2002).

Results from this thesis show that:

- a) Transcription inhibition can be alleviated by interferon treatment and the degradation of MHC I heavy chain (HC) can be reversed by treatment with inhibitors of proteasomes and lysosomes. However, the inhibition of transport of MHC I to the cell surface is irreversible.
- b) E5 is capable of physically interacting with heavy chain. Together with the inhibition of the vacuolar ATPase (due to the interaction between E5 and 16k subunit c), the interaction between E5 and heavy chain is likely to be responsible for retention of MHC I in the Golgi apparatus.
- c) C-terminus deletion mutants of E5 are incapable of either down-regulating surface MHC class I or interacting with heavy chain, establishing that the C-terminus domain of E5 is important in the inhibition of MHC I.
- d) Other two PV E5 proteins, BPV-1 E5 and HPV-16 E5 are able to interact

physically with heavy chains from different alleles. BPV-1 E5 interacts with two equine classical class I heavy chains (EqB2 and EqB4) *in vitro* and the HPV-16 E5 interacts with classical HLA-A1, A2 and B8 *in vitro* and with HLA-A3 *in vivo* (Ashrafi *et al.* 2006b).

e) Like HPV-16 E5, BPV-4 E5 is incapable of down-regulating certain non-classical MHC genes (Araibi *et al.* 2006).

These observations lead to a deeper understanding of the general down-regulation of MHC class I by the E5 proteins. Lack of surface classical MHC class I and presence of non-classical MHC class I in infected cells expressing E5 would allow evasion of cytotoxic T lymphocyte and NK killing and thus establishment of viral infection.

I dedicate this work to my wonderful parents

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Abbreviations

Abbreviation	Definition
ABC	ATP-binding cassette
Ad	Adenovirus
AMF-1	Activation domain modulating factor
AP1	Activator protein-1

ATLL	Adult T-cell leukemia/lymphoma
ATP	Adenosine triphosphate
β_2 -m	β_2 -microglobulin
bp	Base pair
BCA	Bicinchoninic acid
BPV	Bovine papillomavirus
BS	Binding site
BSA	Bovine serum albumin
CBF	CCAAT-box binding factor
CBP	cAMP response element-binding protein
CCRE	Cell cycle repressor element
CDE	Cell cycle-dependent element
CD	Cluster of differentiation
CDK	Cyclin-Dependent Kinase
CIP	Calf Intestinal alkaline Phosphatase
CK	Casein kinase
CMM	Canine microsomal membranes
COP-1	Coat protein complex-1
COPV	Canine Oral Papillomavirus
Co-IP	Co-Immunoprecipitation
CRPV	Cottontail Rabbit Papillomavirus
CSF	Colony stimulating factor
CTL	Cytotoxic T Lymphocyte
CuSO ₄	Copper II sulphate pentahydrate
DEPC	Diethyl Pyrocarbonate

dH ₂ O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DPV	Deer papillomavirus
DTT	Dithiothreitol
<i>E. Coli</i>	Escherichia Coli
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EEPV	European elk papillomavirus
EGF-R	Epidermal Growth Factor Receptor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
Erp57	Thiol-oxidoreductase ⁵⁷
EtBr	Ethidium Bromide
EV	Epidermodysplasia Verruciformis
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal calf serum
FAM	Reporter fluorochrome 6-carboxyfluorescein
FITC	Fluorescein-isothiocyanate
G418	Geneticin, G418-sulphate

GA	Golgi Apparatus
Gar	Glycine-alanine repeat
GAS	Gamma Activated Site
GFP	Green Fluorescent Protein
HaCaT	Human immortalised keratinocyte stable cell line
HC	Heavy chain
HBSS	Hanks balanced salt solution
HBV	Hepatitis B virus
HCMV	Human Cytomegalovirus
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HFK	Human foreskin keratinocyte
Hi-Di	Highly Deionized
HIV	Human Immunodeficiency Virus
hK	Human keratin
HLA	Human Leukocyte Antigen
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
HTLV-1	Human T cell leukaemia virus-1
ICAM	Intercellular adhesion molecule
ICP 47	Infected-cell protein 47
ICSBP	Interferon consensus sequence binding protein
ICTV	International Committee on the Taxonomy of Viruses
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
IP	Immunoprecipitation
IRF	Interferon Regulatory Factor
ISG	Interferon-stimulated gene
ISGF	Interferon-stimulated gene factor
ISRE	Interferon-stimulated response element
JAK	Janus-Activated Kinase
kb	Kilobase pairs
kDa	KiloDalton
KIR	Killer Cell Immunoglobulin-Like Receptor
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
l	Litre
LCR	Long control region
LFA	Lymphocyte function-associated antigen
LMP	Low-molecular Mass Polypeptide
M	Molar
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
Mbp	Mega base pair
MCP	Monocyte chemoattractant protein
MEC	Mammary epithelial cells
MEK	Mitogen-activated protein kinase/extracellular signal- regulated kinase kinase kinase
MES	2-[N-morpholino]ethanesulphonic acid

MCMV	Mouse Cytomegalovirus
MECL	Multicatalytic Endopeptidase Complex-Like
MHC	Major Histocompatibility Complex
mg	Milligram
MIC	MHC class I-chain related
ml	Millilitre
mM	Millimolar
MOPS	3-[N-morpholino]propanesulphonic acid
mRNA	Messenger ribonucleic acid
MW	Molecular Weight
NF-Y	Nuclear factor-Y
nm	Nanometres
NMSC	Non-melanoma skin cancer
NK	Natural killer
°C	Degree centigrade
OD	Optical density (light absorbance)
ORF	Open reading frame
OvPV	Ovine papillomavirus
PA	Proteasome Activator
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline plus 0.1% Tween
PCD	Programmed cell death
PCR	Polymerase Chain Reaction

PDGF-R	Platelet-Derived Growth Factor Receptor
PFA	Paraformaldehyde
pg	picogram
PHA	Phytohemagglutinin
PI-3-K	Phosphoinositide 3-kinase
pmol	Picomole
POMP	Proteasome maturation protein
PV	Papillomavirus
RACK	Receptor for activated C kinases
Rb	Retinoblastoma
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
³⁵ S	Sulphur-35
SDS	Sodium dodecyl sulphate
SIV	Simian immunodeficiency virus
SMN	Survival motor neuron
STAT	Signal Transducers and Activators of Transcription
TAMRA	Quencher fluorochrome 6-carboxy-tetramethyl- rhodamine
TAP	Transporter associated with Antigen Processing
TBE	Tris boric acid ethylenediaminetetracetic acid
TBP	TATA binding protein
TBS	Tris buffered saline
TBS-T	Tris buffered saline plus 0.1% Tween

TCR	T-cell receptor
TE	Tris buffered ethylenediaminetetracetic acid
TGN	Trans-golgi network
T _H	T helper
Tyk	Tyrosine kinase
TM	Transmembrane domain
TNF	Tumour necrosis factor
TR	Texas red
Tris	Tris (hydroxymethyl) aminomethane
TSP-HAM	Tropical spastic paraparesis/HTLV-1-associated myelopathy
Tween 20	Polyoxyethylenesorbitan monolaurate
URR	Upstream regulatory region
UV	Ultraviolet
V	Volts
V0	Membrane-bound proton translocating portion of V-ATPase
V1	Soluble ATP-hydrolysing portion of the V-ATPase
V-ATPase	Vacuolar proton pump adenosine triphosphatase
VLP	Virus-like particle
v/v	Volume per unit volume
WHO	World Health Organization
wt	Wild Type
w/v	Weight per unit volume
µg	Microgram
µl	Microlitre

Single letter amino acid code	
Alanine	Ala (A)
Arginine	Arg (R)
Asparagine	Asn (N)
Aspartic acid	Asp(D)
Cysteine	Cys (C)
Glutamic acid	Glu (E)
Glutamine	Gln (Q)
Glycine	Gly (G)
Histidine	His (H)
Isoleucine	Ile (I)
Leucine	Leu (L)
Lysine	Lys (K)
Methionine	Met (M)
Phenylalanine	Phe (F)
Proline	Pro (P)
Serine	Ser (S)
Threonine	Thr (T)
Tryptophan	Trp (W)
Tyrosine	Tyr (Y)
Valine	Val (V)

CHAPTER 1: INTRODUCTION

1.1 IMMUNE RESPONSE

The mammalian body is susceptible to infection by many pathogens, which must first make contact with the host and then establish a focus of infection in order to cause disease. These pathogens differ greatly in their lifestyles, the structures of their surfaces, and means of pathogenesis, which therefore requires an equally diverse set of defensive responses from the host immune system (Mandell *et al.* 2000). The first phase of host defence consists of those mechanisms that are present and ready to resist an invader at any time. The epithelial surfaces of the body keep pathogens out, and protect against colonization and against viruses and bacteria that enter through specialized cell-surface interactions, by preventing pathogen adherence and by secreting antimicrobial enzymes and peptides (Gibbons R.J. 1992; Podolsky 1999; Ouellette 1999; Krisanaprakornkit *et al.* 2000). Bacteria, viruses, and parasites that overcome this barrier are faced immediately by components of the innate immune system. The innate immune responses rely on the ability of the body to recognize conserved features of pathogens (i.e., LPS, dsDNA, ssRNA) that are absent in the host cell. These pathogen-associated molecules stimulate two types of immune responses, inflammatory responses and phagocytosis by cells such neutrophil and macrophages, which in turn produce chemokines that recruit other inflammatory cells and secrete cytokines that orchestrate the outcome of the inflammatory process. Innate immunity provides a front line of host defence through effector mechanisms that engage the pathogen directly, act immediately on contact with it, and are unaltered in their ability to resist a subsequent challenge with either the same or a different pathogen. These mechanisms often succeed in preventing an infection from becoming established. If

not, they are reinforced through the recruitment and increased production of further effector molecules and cells in a series of induced responses. These induced innate responses often fail to clear the infection. In that case, macrophages and other cells activated in the early innate response help to initiate the development of an adaptive immune response (Janeway *et al.* 2001, 2005).

1.1.1 Innate and adaptive response

Both innate immunity and adaptive immune responses depend upon the activities of white blood cells, or leukocytes. Innate immunity largely involves granulocytes and macrophages among other cell lines (see table 1). Adaptive immune responses depend upon lymphocytes, which provide the lifelong immunity that can follow exposure to disease or vaccination. The innate and adaptive immune systems together provide a remarkably effective defence system. Many infections are handled successfully by the innate immune system and cause no disease; others that cannot be resolved by innate immunity trigger adaptive immunity and are then overcome successfully, followed by lasting immunological memory (Fearon *et al.* 1996; Alan *et al.* 1998; Medzhitov *et al.* 1998; Carroll *et al.* 1998; Ploegh and Watts 1998). The macrophages and neutrophils of the innate immune system provide a first line of defence against many common microorganisms and are essential for the control of common bacterial infections. However, they cannot always eliminate infectious organisms, and there are some pathogens that they cannot recognize. The lymphocytes of the adaptive immune system have evolved to provide a more versatile means of defence which, in addition, provides increased protection against subsequent reinfection with the same pathogen (Picker and Butcher 1992; Butcher and Picker 1996; Paul 1998; Mandell *et al.* 2000).

Pathogens are accessible to antibodies only in the blood and the extracellular spaces. However, some bacterial pathogens and parasites, and all viruses, replicate inside cells where they cannot be detected by antibodies. The destruction of these invaders is the function of the T lymphocytes, or T cells, which are responsible for the cell-mediated immune responses of adaptive immunity. Cell-mediated reactions depend on direct interactions between T lymphocytes and cells bearing the antigen that the T cells recognize. The actions of cytotoxic T cells (CTL) are the most direct. These recognize any of the body's cells that are infected with viruses, which replicate inside cells, using the biosynthetic machinery of the cell itself. The replicating virus eventually kills the cell, releasing new virus particles. Antigens derived from the replicating virus are, however, displayed on the surface of infected cells, where they are recognized by cytotoxic T cells. These cells can then control the infection by killing the infected cell before viral replication is complete (Paul 1998; Mandell *et al.* 2000).

All the effects of T lymphocytes depend upon interactions with target cells containing foreign proteins. Cytotoxic T cells and T helper type-1 (T_H1) cells interact with antigens produced by pathogens that have infected the target cell or that have been ingested by it. T helper Type-2 (T_H2) cells, in contrast, recognize and interact with B cells that have bound and internalized foreign antigen by means of their surface immunoglobulin. In all cases, T cells recognize their targets by detecting peptide fragments derived from the foreign proteins, after these peptides have been captured by specialized molecules in the host cell and displayed by them at the cell surface (Figure 1) (Paul 1998; Janeway *et al.* 2001).

Innate immunity elements	Function
Complement components	Many distinct plasma proteins that can be activated by pathogens and induce a series of inflammatory responses
Dendritic cells	Phagocytic cells that when immature can take up pathogens; when mature they act as antigen- presenting cells to T cells, initiating adaptive immune responses
Eosinophils	Are thought to be involved in the killing of antibody-coated parasites (worms)
Macrophages	Primary phagocytic cells that engulf pathogens and destroy them in intracellular vesicles; also present antigens to T cells and can activate them
Mast cells	Tissue cells that trigger a local inflammatory response by releasing substances that act on local blood vessels
Neutrophils	Primary phagocytic cells that engulf pathogens and destroy them in intracellular vesicles
NLRs (nucleotide-binding oligomerization domain (Nod)-like receptors)	Family of receptors that sense pathogens in the cytosol and induce host defence signaling pathways upon activation
NK cells	Activated in response to interferons or macrophage-derived cytokines. Killing of virus infected and tumour cells while the adaptive immune response is generating antigen-specific cytotoxic T cells
TLRs (Toll-like receptors)	Family of receptors for many bacterial and viral components (i.e.LPS, dsRNA, ssRNA etc.) expressed by macrophages and dendritic cells that mediate extracellular recognition of pathogens

Table 1 The elements of innate immunity

Summary adapted from Franchi et al., 2006, Kawai and Akira 2006, Janeway et al., 2005.

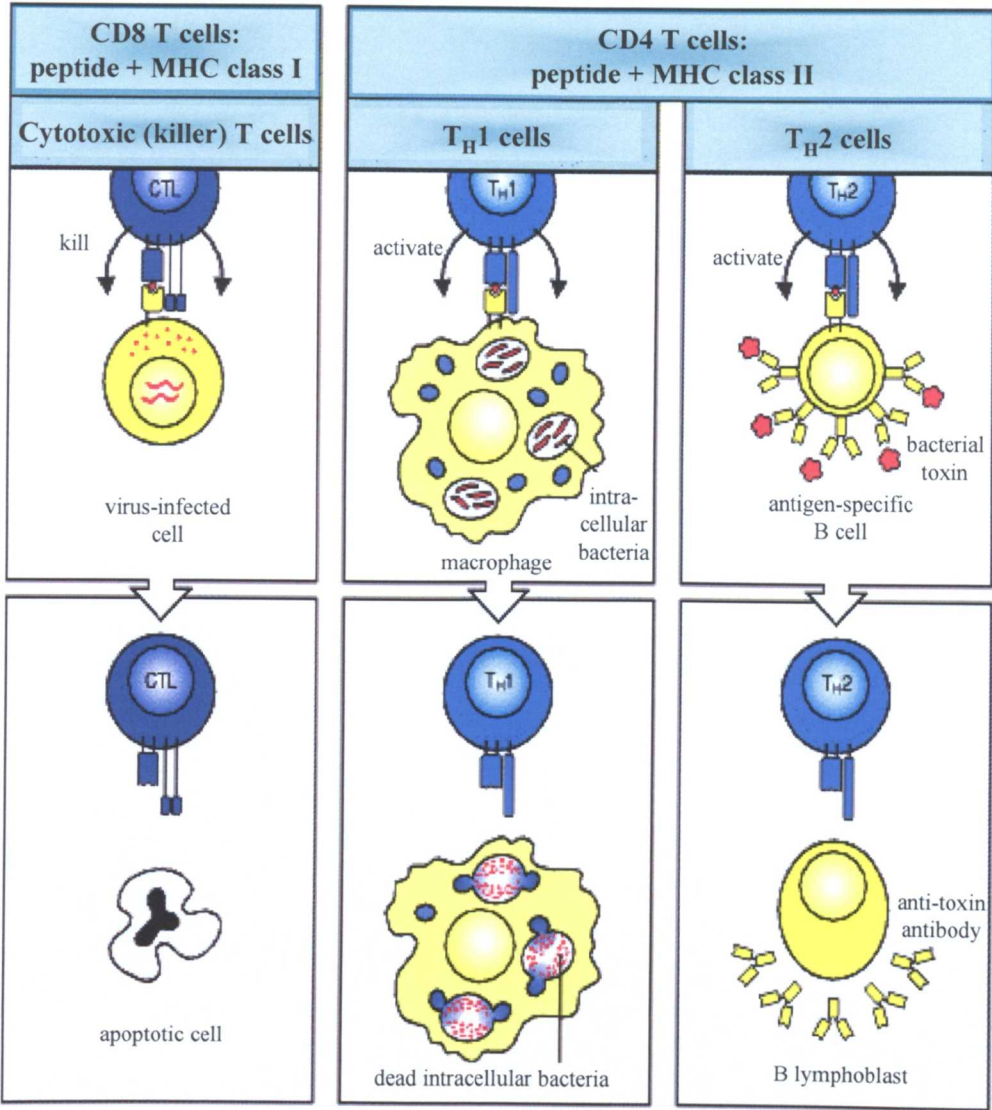


Figure 1. CD8 and CD4 T cells. The CD8 cytotoxic T cells (left panels) interact with antigenic peptides produced by cytosolic pathogens and are presented by MHC class I molecules at the cell surface of the infected cells. TH1 cells (middle panels) and TH2 cells (right panels) which express the CD4 co-receptor recognize fragments of antigenic peptides derived from pathogens taken up into intracellular vesicles and displayed at the cell surface by MHC class II molecules.

TH1 are able to activate macrophages and they can also activate B cells to produce antibodies belonging to certain IgG subclasses. TH2 cells stimulate B cells to differentiate and produce immunoglobulins of all other types, and also activate naive B cells to proliferate and secrete IgM. The different types of immunoglobulin together form the effector molecules of the humoral immune response. (Picture courtesy of Charles A Janeway, Paul Travers, Mark Walport, Mark Shlomchik in Immunobiology © 2001 by Garland Publishing).

The molecules that display peptide antigen to T cells are membrane glycoproteins encoded in a cluster of genes named major histocompatibility complex, abbreviated to MHC.

There are two types of MHC molecule, called MHC class I and MHC class II (figure 1.1). These differ in several subtle ways but share most of their major structural features (Bjorkman *et al.* 1987a; Brown *et al.* 1993; Jones 1997). The most important of these is formed by the two outer extracellular domains of the molecule, which combine to create a long cleft in which a single peptide fragment is trapped during the synthesis and assembly of the MHC molecule inside the cell (Madden *et al.* 1992; Stern and Wiley 1994; Murthy and Stern 1997). The MHC molecule bearing its cargo of peptide is then transported to the cell surface, where it displays the peptide to T cells. The antigen receptors of T lymphocytes are specialized to recognize a foreign antigenic peptide fragment bound to an MHC molecule. A T cell with a receptor specific for the complex formed between that particular foreign peptide and MHC molecule can then recognize and respond to the antigen-presenting cell (Paul 1998).

The most important differences between the two classes of MHC molecule lie not in their structure but in the source of the peptides that they trap and carry to the cell surface. MHC class I molecules collect peptides derived from proteins synthesized in the cytosol, and are thus able to display fragments of viral proteins on the cell surface. MHC class II molecules bind peptides derived from proteins in intracellular vesicles, and thus display peptides derived from pathogens living in macrophage vesicles or internalized by phagocytic cells and B cells (Morrison *et al.* 1986).

Having reached the cell surface with their peptide cargo, the two classes of MHC molecule are recognized by different functional classes of T cell. MHC class I

molecules bearing viral peptides are recognized by cytotoxic T cells, which then kill the infected cell; MHC class II molecules bearing peptides derived from pathogens taken up into vesicles are recognized by helper T cells T_H1 , which activate macrophages and can release cytokine and chemokines or helper T cells T_H2 that activate B cells.

The antigen-specific activation of these effector T cells is aided by co-receptors that distinguish between the two classes of MHC molecule; cytotoxic cells express the CD8 co-receptor that binds MHC class I molecules, whereas T_H1 and T_H2 cells express the CD4 co-receptor with specificity for MHC class II molecules. On recognizing their targets, the three types of T cell are stimulated to release different sets of effector molecules. These effector molecules include many cytokines, which have a crucial role in the clonal expansion of lymphocytes as well as in innate immune responses and in the effector actions of most immune cells (Paul 1998; Janeway *et al.* 2001).

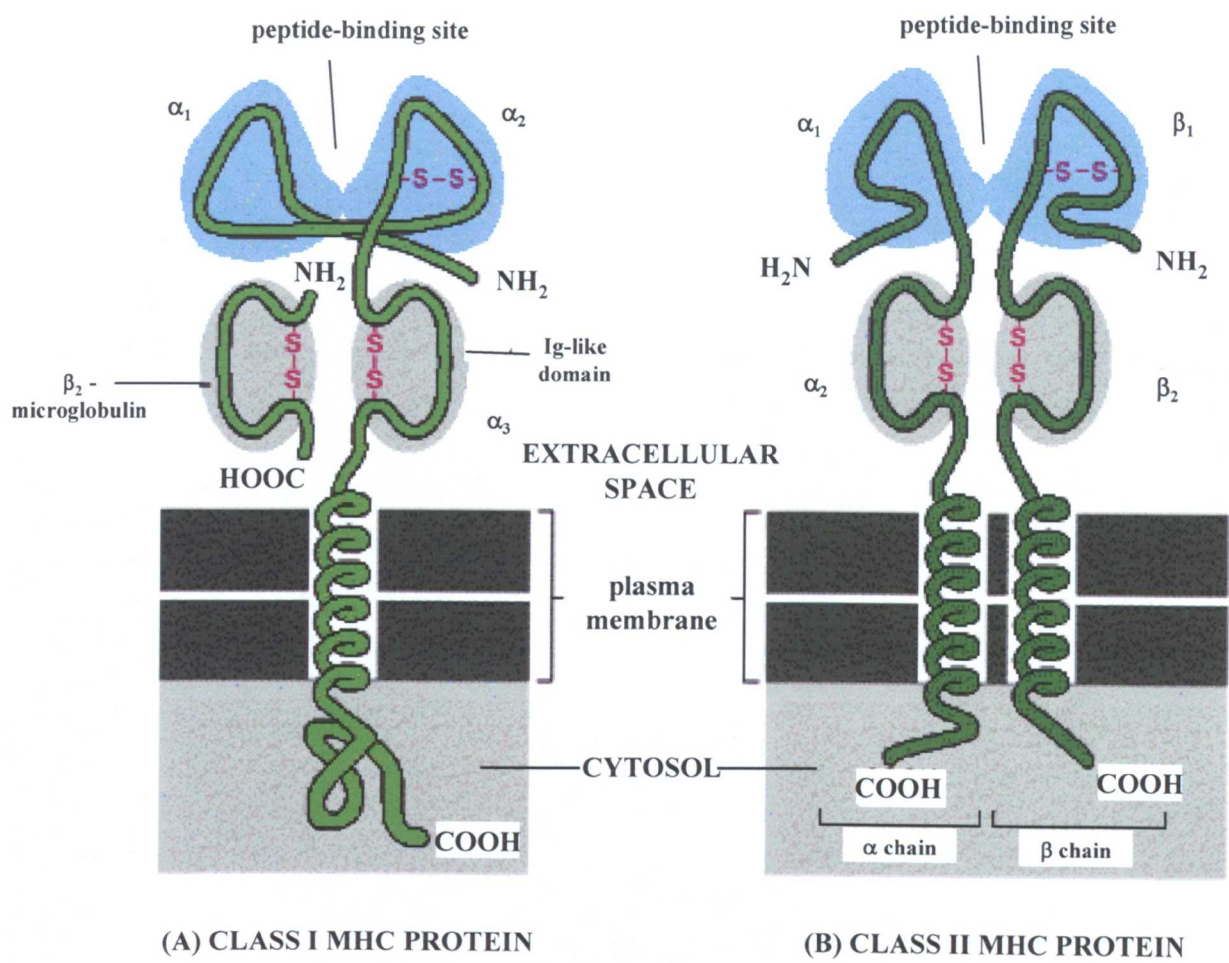


Fig 1.1 MHC class I and class II proteins. (A) The class I MHC proteins consists of an α chain with an extracellular region composed by three domains, α_1 , α_2 and α_3 which are encoded by separate exons. The α_1 and α_2 domains are highly polymorphic. The α_3 domain is Ig-like. β_2 -microglobulin is a small non glycosylated protein that is associated noncovalently with the α chain. (B) The class II MHC proteins consist of two chains α and β which are highly polymorphic in the α_1 and β_1 domains whereas the α_2 and β_2 domains are Ig-like. The α_1 and α_2 domains of the class I proteins and α_1 and β_1 domains of class II proteins interact to create a long cleft (shaded in blue) in which peptide fragments of foreign proteins are trapped and then presented to T cells. (Picture courtesy of Molecular Biology of The Cell © 2002 by Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter).

1.1.2 CD4 and CD8 co-receptors

T cells fall into two major classes that have different effector functions. The two classes are distinguished by the expression of the cell-surface proteins CD4 and CD8 (Swain 1983; Norment *et al.* 1988; Salter *et al.* 1990; Cammarota *et al.* 1992; König *et al.* 1992).

CD4 is a single-chain molecule composed of four immunoglobulin-like domains (Figure 1.2). The first two domains (D₁ and D₂) of the CD4 molecule are packed tightly together to form a rigid rod some 60 Å long, which is joined by a flexible hinge to a similar rod formed by the third and fourth domains (D₃ and D₄) (Ryu *et al.* 1990; Wang *et al.* 1990; Wu *et al.* 1997). CD4 binds MHC class II molecules through a region that is located mainly on a lateral face of the first domain, D₁. Because CD4 binds to a site on the β domain of the MHC class II molecule that is well away from the site where the T-cell receptor binds, the CD4 molecule and the T-cell receptor can bind the same peptide:MHC class II complex (Janeway 1992; Reich *et al.* 1997). When CD4 and the T-cell receptor can simultaneously bind to the same MHC class II:peptide complex, the sensitivity of a T cell to antigen presented by MHC class II molecules is markedly increased; the T-cell in this case requires 100-fold less antigen for activation (Zamoyska 1998; Gao G.F. *et al.* 2002).

The CD8 molecule is a disulfide-linked heterodimer consisting of an α and a β chain, each containing a single immunoglobulin-like domain linked to the membrane by a segment of extended polypeptide chain (Figure 1.2) (Parnes 1989; Leahy *et al.* 1992). This segment is extensively glycosylated, which is thought to be important in maintaining this polypeptide in an extended conformation and protecting it from cleavage by proteases. CD8α chains can also form homo-dimers, although these are

not found when the CD8 β chains are present. CD8 binds weakly to an invariant site in the α_3 domain of an MHC class I molecule, which is equivalent to the site in MHC class II molecules to which CD4 binds (Sun *et al.* 1995). Although only the interaction of the CD8 α homodimer with MHC class I is so far known in detail (Gao *et al.* 1997), it is clear from this that the MHC class I binding site of the CD8 α : β heterodimer will be formed by the interaction of the CD8 α and β chains. In addition, CD8 (most probably through the α chain) interacts with residues in the base of the α_2 domain of the MHC class I molecule (Sun *et al.* 1995; Gao *et al.* 2000b). Binding in this way, CD8 leaves the upper surface of the MHC class I molecule exposed and free to interact simultaneously with a T-cell receptor (Gao *et al.* 2000a; Gao *et al.* 2002). And as with CD4, the presence of CD8 increases the sensitivity of T cells to antigen presented by MHC class I molecules by about 100-fold. Thus, CD4 and CD8 have similar functions and bind to the same approximate location in MHC class I and MHC class II molecules even though the structures of the two co-receptor proteins are only distantly related (Gao *et al.* 2002).

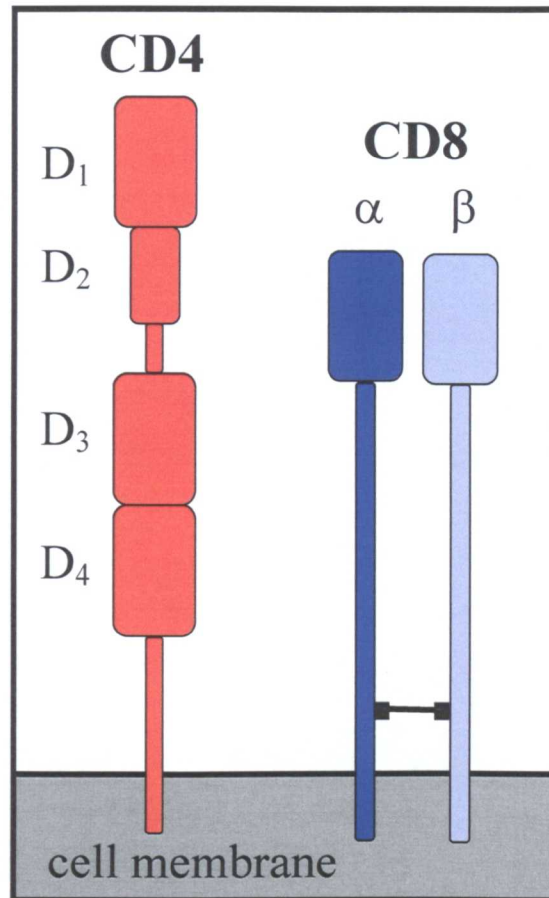


Figure 1.2 The CD4 and CD8 co-receptor molecules. CD4 is a single-chain molecule composed of four immunoglobulin-like domains. The D₁ and D₂ domains are packed tightly together to form a rigid rodlike structure that is linked to the carboxy-terminal domains (D₃ and D₄) by a flexible link. It is thought that the binding site for MHC class II molecules involve both the D₁ and D₂ domains. CD8 consists of an α and a β chain covalently linked by a disulfide bond; it can also exist as an homodimer of α chains. CD8α and CD8β immunoglobulin-like domains are linked to the membrane by an extended polypeptide chain. The binding site for MHC class I molecules is thought to involve the CD8α chain (Picture courtesy of Charles A Janeway, Paul Travers, Mark Walport, Mark Shlomchik in Immunobiology © 2001 by Garland Publishing).

1.1.3 The Major Histocompatibility Complex Class I (MHC I)

The major histocompatibility complex (MHC) is located on chromosome 6 in humans, chromosome 17 in mouse and chromosome 23 in cattle. The MHC spans approximately 4 Mbp of the human genome, 1.5 Mbp in mice and approximately 2.5 Mbp in cattle (Gellin *et al.* 2000). In mammals it contains more than 260 genes that in humans are called Human Leukocyte Antigen or HLA genes, as they were first discovered through antigenic differences between white blood cells from different individuals; in mouse they are known as the H-2 genes and in cattle as bovine leukocyte antigen (BoLA) genes (Beisel *et al.* 1980; Demant *et al.* 1981; The MHC sequencing consortium 1999; Beck *et al.* 2000; Horton *et al.* 2004). The structure and organization of the MHC genes of cattle, are very similar to those of the human. The major difference between their organization is that the BoLA class II loci are found in two separate regions of the chromosome rather than in a single cluster of genes as seen in most mammals (Andersson *et al.* 1988; van Eijk *et al.* 1993; Childers *et al.* 2006). The mature MHC class I complexes consist of three noncovalently associated components: a 45-kDa heavy chain (HC); a 12-kDa light chain, β_2 -microglobulin (β_2 -m); and a short peptide antigen (Jones 1997). There are three class Ia loci in humans called HLA-A, B, and C; in mice, H-2K, H-2D, and H2L (Ploegh *et al.* 1981). There are also many class Ib (Shawar *et al.* 1994) and MIC (MHC class I-chain related) loci (Bahram *et al.* 1994). The class Ia HC genes are extremely polymorphic (Parham *et al.* 1995). These genes encode type I glycoproteins of approximately 340 amino acids, consisting of a cytoplasmic region (about 30 residues), a transmembrane region, and an extracellular region composed of three domains ($\alpha 1$, $\alpha 2$, and $\alpha 3$) with one to three N-linked glycosylation sites (Bjorkman *et al.* 1987a). β_2 -m is a nonglycosylated protein of about 100 amino acids encoded on chromosome 15 in humans and 2 in

mice. This protein has no transmembrane domain and remains associated with cells by interacting with the extracellular region of HC. The structure of a number of MHC I molecules has been solved (Bjorkman *et al.* 1987a; Bjorkman *et al.* 1987b; Wilson *et al.* 1993; Young *et al.* 1995). β_2 -m makes extensive contacts with all three domains of the HC (Bjorkman *et al.* 1987a). Because of this, the conformation of the HC is very dependent on the presence or absence of β_2 -m. The membrane-proximal $\alpha 3$ region of the HC is an immunoglobulin-like domain that contains a binding site for the CD8 receptor on CTL. The $\alpha 1$ and $\alpha 2$ domains, which are distal to the membrane and interact with the T cell receptor on CTL, fold together to form a groove that binds and displays peptides. A beta-pleated sheet forms the base of the cleft, and the walls are made of two alpha helices. The allelic polymorphisms in HC primarily occur in those residues in and around this cleft, and in this manner, they alter the peptide-binding specificity of the class I molecules (Bjorkman *et al.* 1987b; Parham *et al.* 1995; York *et al.* 1996).

The solution of the crystal structure of the class I proteins also provided insight into the problem of how a single type of molecule could bind and present so many different peptides. Many of the molecular interactions are with the peptide main chain atoms and amino and carboxy termini, which are features common to all peptides. The groove is generally long enough to accommodate 8 or 9 residues in an extended conformation (Madden *et al.* 1992). Longer peptides can also fit by bulging partly out of the groove or by zigzagging within the cleft (Madden *et al.* 1993; Madden 1995). In some cases the ends of the peptide may extend out of the groove, although this decreases the stability of interaction (Chen *et al.* 1994; Collins *et al.* 1994).

1.1.3.1 The antigen processing and presentation

An effector cytotoxic T cell kills a virus-infected cell when it recognizes fragments of viral proteins bound to MHC class I on the surface of the infected cell (Figure 1.3). The major pathway for degrading proteins in the cytosol is ATP-dependent and highly conserved from yeast to mammals (Goldberg *et al.* 1976). In this process proteins are hydrolyzed to oligopeptides by a large multicatalytic proteolytic particle, the proteasome (Rechsteiner *et al.* 1993; Peters 1994). One form of the proteasome is a 20S (700 kDa) cylindrical particle composed of four rings (Lowe *et al.* 1995). Another form of the proteasome is a 26S (1500 kDa) particle, which contains as its proteolytic core the 20S structure associated with many additional subunits that regulate its activity (Rechsteiner *et al.* 1993; Peters 1994). This larger particle degrades some protein substrates directly in an ATP-dependent manner. In addition, the 26S structure binds polyubiquitin chains (Deveraux *et al.* 1994) and is responsible for degrading ubiquitin-conjugated proteins. The degradation of many cellular proteins is initiated by their modification with the small polypeptide ubiquitin that is attached via an isopeptide bond to lysines on the substrate and on other ubiquitins (Ciechanover 1994; Hershko and Ciechanover 1998).

Many unfolded proteins and polypeptides, can be ubiquitinated and degraded by the proteasome. Thus, is not surprising that the proteasome is the major source of peptide supply to class I molecules (Rock and Goldberg 1999).

It has been shown that when cells are stimulated with IFN- γ , a cytokine that is commonly produced after virus infection, the synthesis of three proteasome subunits, low-molecular mass polypeptide-2 (LMP2), -7 (LMP7), and multicatalytic endopeptidase complex-like-1 (MECL-1) are induced (Brown *et al.* 1991; Nandi *et al.* 1996; Rock and Goldberg 1999). Furthermore the genes encoding LMP2 and LMP7

are MHC-linked, suggesting that these subunits of the proteasome have co-evolved with MHC class I molecules (Glynne *et al.* 1991; Kelly *et al.* 1991).

Proteasomes that contain IFN- γ inducible subunits are termed immunoproteasomes and represent the predominant form found in antigen presenting cells (APCs) and inflamed tissues (Stohwasser *et al.* 1997; Macagno *et al.* 1999). Data suggest that IFN- γ induces formation of immunoproteasomes and the synthesis of the proteasome activator PA28 (Nandi *et al.* 1996; Macagno *et al.* 1999; Strehl *et al.* 2005). Both alter the proteolytic properties of the proteasome complex and enhance proteosomal function in antigen presentation (Aki *et al.* 1994; Eleuteri *et al.* 1997; Hendil *et al.* 1998; Kloetzel 2004).

Some peptides produced in the cytosol are further degraded by endopeptidases and aminopeptidases into amino acids, which may be used for protein synthesis or energy production (Hershko and Ciechanover 1998). A fraction of these peptides must pass into the lumen of the ER before they can bind to MHC I molecules that are assembled in this organelle. This process is made possible by TAP (transporter associated with antigen presentation) that is a member of the ATP-binding cassette (ABC) family of transporters, which share a common architecture and require ATP for their transport activity (Monaco 1992; Townsend *et al.* 1993; Reits *et al.* 2000). The members of this family transport across cell membranes a wide range of molecules, ranging from ions to large proteins. They have either 6 or 12 membrane spanning domains; those with 6 transmembrane domains, such as the TAP proteins, function as heterodimers or homodimers. The two subunits of TAP are approximately 76 (TAP1) and 70 (TAP2) kDa and are noncovalently associated. Although one study suggests that TAP1 might also be able to function as a homodimer (Gabathuler *et al.* 1994), TAP1 and TAP2

generally seem nonfunctional when expressed alone (Meyer T.H. *et al.* 1994; Russ *et al.* 1995). Immunoelectron microscopy and confocal analysis have localized TAP to the ER (Russ *et al.* 1995) and the *cis*-Golgi (Kleijmeer *et al.* 1992). This subcellular distribution is consistent with the location in cells where MHC molecules bind peptides. The sequence of both the TAP1 and TAP2 subunits shows consensus ATP binding sites in the cytoplasmic domain, near the C-terminus. The peptide-binding site is also in the cytoplasmic region and is probably formed by both subunits (van Endert *et al.* 1995) (van Endert *et al.* 1994). Peptide binds to the TAP complex independently of ATP (van Endert *et al.* 1994) while peptide translocation is ATP-dependent (Androlewicz *et al.* 1993).

TAP effectively binds peptides ranging from 8 to 13 residues in length, but peptides of up to 40 residues can be translocated (Pamer and Cresswell 1998).

The finding that many of the transported peptides are longer than the optimal length (8-10 residues) for binding to MHC molecules is consistent with the notion that some peptides require further N-terminal trimming in the ER (Stoltze *et al.* 2000). In mice, MHC I molecules show preferences for binding peptides containing hydrophobic residues at the C-terminus, and human MHC I mainly binds peptides with hydrophobic and basic C-termini; the TAP transporters in each case show a similar bias (Schumacher *et al.* 1994; Momburg *et al.* 1994).

Studies with TAP-deficient cell lines (Townsend *et al.* 1989), TAP1-deficient mice (Van Kaer *et al.* 1992) and TAP-deficient humans (de la Salle H. *et al.* 1994) have provided strong evidence that TAP is the major source of peptide supply to the ER lumen.

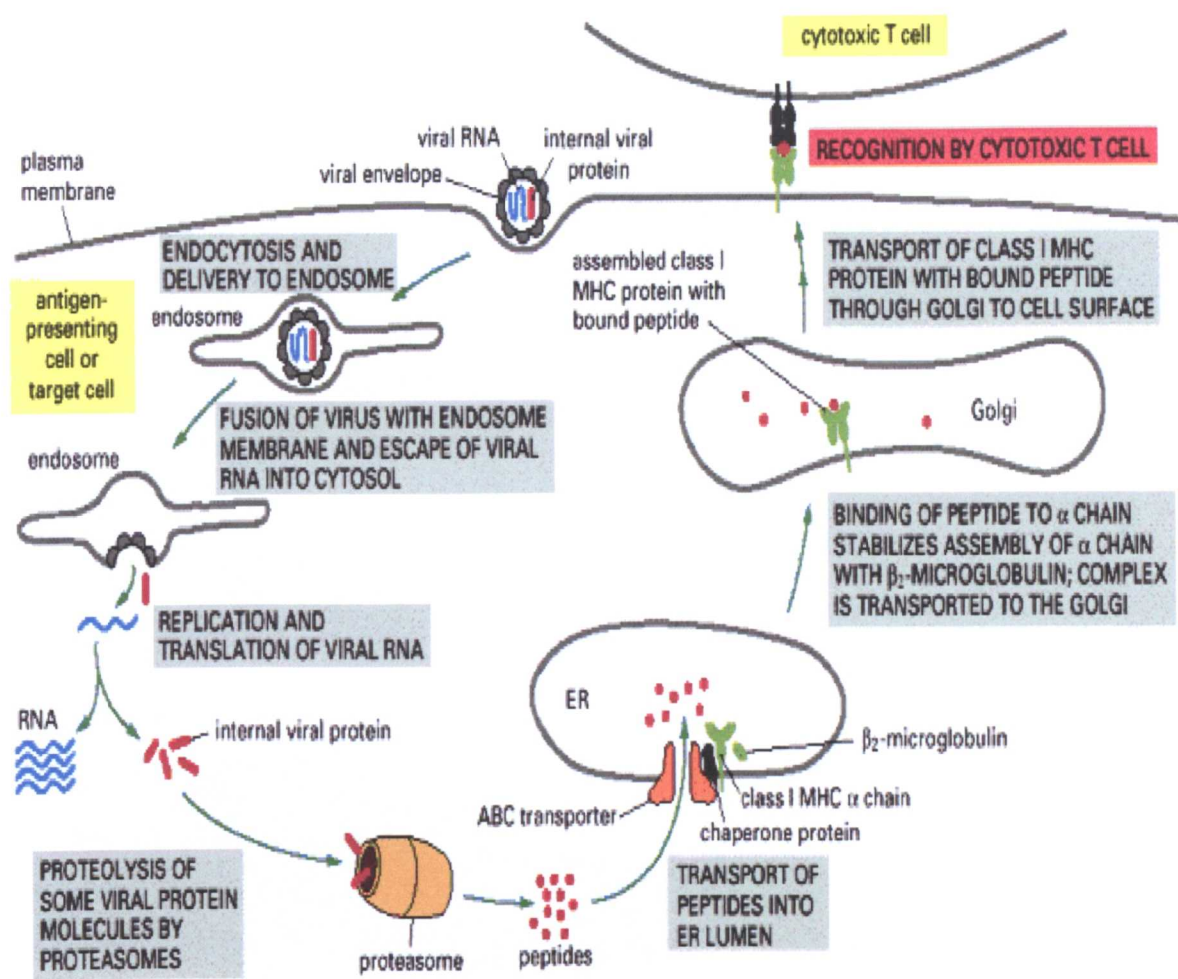


Figure 1.3 Viral protein processing for presentation to CTL cells.

After the entry of a virus in a target cell (here is shown an enveloped RNA virus) some of the viral proteins synthesized are degraded by the proteasome. A fraction of these peptides are then transported into the lumen of the ER where they can bind to the MHC class I associated in the loading complex. This consists of several chaperone proteins only one of which is shown in black. After peptide binding the MHC I loaded complex is transported through the GA to the cell surface for presentation to cytotoxic T cells. (Picture courtesy of Molecular Biology of The Cell © 2002 by Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter)

The MHC I heavy and light chains are synthesized separately on the rough ER and are cotranslationally transported into the ER. The loading of MHC class I molecules with peptides in the ER is a highly regulated process that involves a variety of accessory proteins (Figure 1.4) (Pamer and Cresswell 1998; Cresswell 2000; Van Kaer 2001). The initial steps of class I assembly are largely controlled by chaperones with general housekeeping functions, whereas dedicated proteins participate in the later stages of class I assembly (Van Kaer 2001). The initial steps of class I assembly are controlled by the lectin-like chaperones calnexin and calreticulin, both of which recognize monoglucosylated N-linked glycans (Pamer and Cresswell 1998; Van Kaer 2001). Soon after their synthesis class I heavy chains bind with calnexin. Binding of class I heavy chain with β_2 -m induces the exchange of calnexin for the chaperone calreticulin, at least in human cells. In murine cells, interaction of calnexin with class I heavy chains may persist after β_2 -m binding, but the significance of this species difference remains unknown. Although the association of calnexin with class I heavy chains is undisputed, its importance for class I assembly is unclear because this process proceeds normally in a human calnexin-deficient cell line (Sadasivan *et al.* 1995). One possible explanation for this finding is that, in human cells, other chaperones such as immunoglobulin heavy chain binding protein (BiP) can compensate for the loss of calnexin. There is indeed evidence that BiP binds with human class I heavy chains during the early steps of class I assembly (Nossner and Parham 1995).

Class I heterodimers that bind with calreticulin are typically found in association with a variety of other ER-resident proteins, including Erp57, the two subunits of the TAP peptide transporter, and TAP-associated glycoprotein (tapasin) (Cresswell 2000;

Grande *et al.* 2001). This multifactorial protein complex is called the MHC class I peptide-loading complex.

Tapasin is a transmembrane protein with an ER retention signal in its cytoplasmic domain (Cresswell 2000), is encoded by an MHC-linked gene, and its expression is up-regulated by IFN- γ . It has been suggested that tapasin is in fact an MHC class I-like protein (Mayer and Klein 2001). Tapasin forms a physical link between the two subunits of the TAP transporter and the class I heterodimers (Suh *et al.* 1994; Ortmann *et al.* 1997).

Erp57 is a member of the protein disulfide isomerase family and mediates disulfide bond rearrangements in glycoproteins to which it is recruited by calnexin or calreticulin (Cresswell *et al.* 1999), moreover it interacts with tapasin, via an interchain disulfide bond (Dick *et al.* 2002).

Emerging evidence indicates that TAP heterodimers initially interact with tapasin and Erp57, assisted by calnexin. This intermediate complex then binds with class I/ β_2 -m heterodimers, with release of calnexin and recruitment of calreticulin, to generate the mature loading complex (Diedrich *et al.* 2001). It has been shown that Erp57 and tapasin are covalently linked by a disulfide bond within the loading complex, and that this interaction is critical for complete oxidation of class I heavy chain (Dick *et al.* 2002).

The role of calreticulin in class I assembly was recently evaluated with fibroblast cells derived from mouse embryos that carry a lethal, homozygous mutation in their calreticulin gene. Class I molecules in these cells fail to load with optimal peptide ligands but these molecules are released into the secretory pathway at a normal or even enhanced rate. Calreticulin-deficient cells have moderately reduced class I surface levels and are defective in antigen presentation to class I-restricted CTLs (Gao

et al. 2002). These findings indicate that tapasin, Erp57 and calreticulin play cooperative and non-redundant roles in class I assembly.

Peptide translocation into the ER by TAP induces the release of class I/ β_2 -m heterodimers from the peptide-loading complex (Pamer and Cresswell 1998; Cresswell *et al.* 2005). In mouse cells it has been shown that release from calnexin occurs after dissociation from TAP, so it appears that calnexin ultimately determines if a class I molecule is to be exported from the ER (Suh *et al.* 1996; Marguet *et al.* 1999). Exit of peptide-loaded class I molecules from the ER occurs by selective mechanism involving association with transport receptors (Herrmann *et al.* 1999; Spiliotis *et al.* 2001).

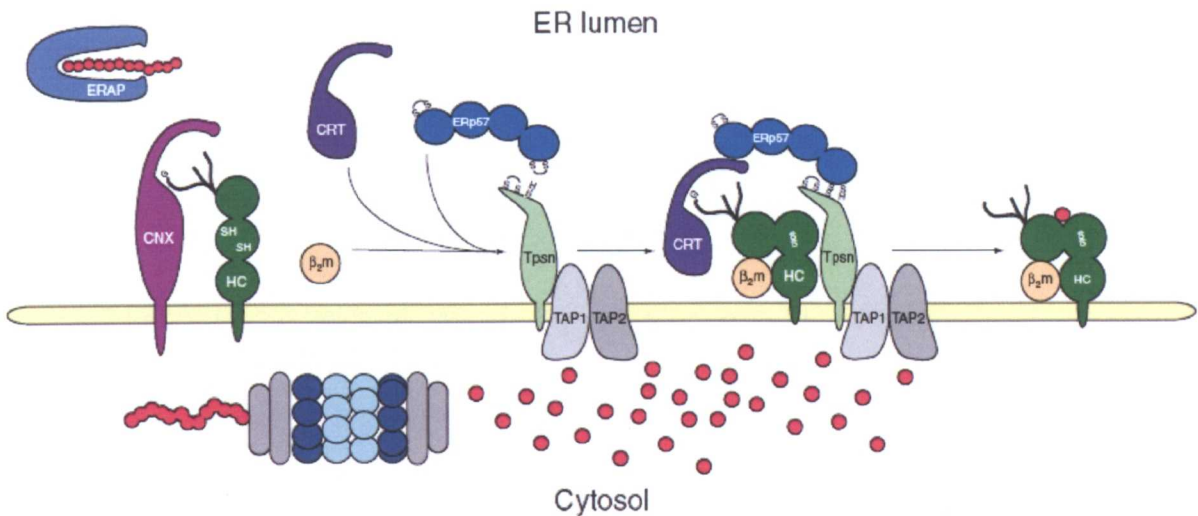


Fig. 1.4 The major histocompatibility complex (MHC) class I assembly and loading pathway. The initial step of MHC class I complex assembly starts with the association between the MHC class I heavy chain and the chaperone calnexin (CNX). After binding the β_2 -microglobulin (β_2 -m) the folded MHC heavy chain becomes associated with the peptide-loading complex, consisting of the transporter associated with antigen presentation (TAP), tapasin, Erp57 and calreticulin (CRT). The peptides derived from degradation of viral proteins in the cytosol are then transported into the endoplasmic reticulum (ER) lumen via TAP. Some peptides in the ER may require further trimming by an ER-associated aminopeptidase (ERAP-1) to reach the optimal length of 8-10 amino acids necessary for binding to MHC I molecules. The binding of a peptide of the appropriate sequence to the class I molecule initiates its dissociation from the peptide-loading complex. (Picture courtesy of Cresswell *et al.* 2005, Immunological Reviews vol. 207:145-157)

1.1.4 NK cells and non-classical MHC/HLA

Natural Killer cells (NK) develop in the bone marrow from the common lymphoid progenitor cell and circulate in the blood.

The mechanism of NK cell killing is the same as that used by cytotoxic T cells generated in an adaptive immune response; cytotoxic granules are released onto the surface of the bound target cell, and the proteins they contain penetrate the cell membrane and induce programmed cell death. NK cells also secrete cytokines such as interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) during infection and inflammation (Salazar-Mather *et al.* 2000). NK cells are activated in response to interferon- α (IFN- α) and interferon- β (IFN- β) or macrophage-derived cytokines such as interleukin-2 (IL-2), which is one of the cytokines produced early in many infections (Biron *et al.* 1999). Activated NK cells serve to contain virus infections while the adaptive immune response generates antigen-specific cytotoxic T cells that can clear the infection.

NK cells have two types of surface receptor that control their cytotoxic activity. One type is an 'activating receptor' which triggers killing by the NK cells. Several types of receptor provide this activation signal, including calcium-binding C-type lectins that recognize a wide variety of carbohydrate ligands present on many cells. In human the identified cytotoxic receptors are NKp30, NKp44, NKp46 (Bianchini *et al.* 2001) and in mouse Ly49D and Ly49H (Smith *et al.* 2001).

A second set of receptors inhibit activation and prevent NK cells from killing normal host cells. These 'inhibitory receptors' are specific for MHC class I alleles, which helps to explain why NK cells selectively kill target cell bearing low level of MHC class I molecules. Various inhibitory receptors are consistently expressed by subsets of NK cells including killer-cell immunoglobulin-like receptors (KIRs),

immunoglobulin-like inhibitory receptors (ILT) and the lectin-like heterodimer CD94-NKG2 (Biassoni *et al.* 2001). Also found in mice the CD94-NKG2 receptor interacts with non-polymorphic MHC-like molecules, HLA-E in human and Qa-1 in mice (Renard *et al.* 1997; Lanier 1998; Vales-Gomez *et al.* 2000a).

In fact, in addition to the highly polymorphic “classical” MHC class I and class II genes, there are many genes encoding MHC class I-type molecules that show little polymorphism. These genes have been termed MHC class Ib genes (Bahram *et al.* 1994). Some class Ib genes, for example the members of the MIC gene family, are under a different regulatory control from the classical MHC class I genes and are induced in response to cellular stress. There are five MIC genes, but only two, MIC A and MIC B, are expressed and produce protein products. They are expressed in fibroblasts and epithelial cells, particularly in intestinal epithelial cells, and may play a part in innate immunity (Groh *et al.* 1998).

The MIC A and MIC B molecules are recognized by a receptor that is present on NK cells and is capable of activating these cells to kill MIC-expressing targets. The MIC receptor is composed of two chains. One is NKG2D, an ‘activating’ member of the NKG2 family of NK-cell receptors the other is a protein called DAP10, which transmits the signal into the interior of the cell by interacting with an activating intracellular protein tyrosine kinase (Wu *et al.* 1999; Moretta *et al.* 2001).

Other MHC class Ib molecules may inhibit cell killing by NK cells. Such a role has been suggested for the MHC class Ib molecule HLA-G, which is expressed on foetus-derived placental cells that migrate into the uterine wall. These cells express no classical MHC class I molecules and cannot be recognized by CD8⁺T cells but, unlike other cells lacking classical MHC class I molecules, they are not killed by NK cells (Verma *et al.* 1997; Pende *et al.* 1997; Braud *et al.* 1999a).

Another MHC class Ib molecule, HLA-E, also has a specialized role in cell recognition by NK cells. HLA-E binds a very restricted subset of peptides, derived from the leader sequence of other HLA class I molecules. These peptides/HLA-E complexes can bind to the receptor NKG2A, which is present on NK cells in a complex with the cell-surface molecule CD94. NKG2A is an inhibitory member of the NKG2 family, and on stimulation inhibits the cytotoxic activity of the NK cell (Braud *et al.* 1998) (Braud and McMichael 1999b) (Lopez-Botet *et al.* 2000; Vales-Gomez *et al.* 2000a; Vales-Gomez *et al.* 2000b). Thus a cell that expresses either HLA-E or HLA-G is not killed by NK cells.

1.2 PAPILLOMAVIRUSES

The Papillomaviruses (PVs) had been originally grouped with the polyomaviruses in one family, the *Papovaviridae*. This was based on similar, nonenveloped capsids and the common circular double-stranded DNA genomes. As it was later recognized that the two virus groups have different genomes sizes, completely different genome organizations, and no major nucleotide or aminoacid sequence similarities, they are now officially recognized by the International Committee on the Taxonomy of Viruses (ICTV) as two separate families, *Papillomaviridae* and *Polyomaviridae* (de Villiers *et al.* 2004).

One hundred eighteen papillomavirus type have been completely described, and a yet higher number of presumed new types have been detected by preliminary data (de Villiers *et al.* 2004).

PVs are a diverse group of viruses that have been found in more than 20 different mammalian species, as well as in birds and reptiles. They are strictly species specific and are not known to cross the species barriers with the exception of Bovine Papillomaviruses (BPVs), which can infect horses and induce equine sarcoid (Olson and Cook 1951; Ragland *et al.* 1969; Lancaster *et al.* 1977; Chambers *et al.* 2003a; Chambers *et al.* 2003b).

PVs are non enveloped viruses with icosahedral capsids that replicate their genomes within the nuclei of infected host cells. They have circular double-stranded DNA genomes with sizes close to 8Kb. In spite of their small size, their molecular biology is very complex. Briefly, three oncoproteins, E5, E6, and E7, modulate the transformation process, two regulatory proteins, E1 and E2, modulate transcription and replication, and three late proteins of which L1 and L2, compose the viral capsid

and E1^{E4} has several possible functions late in infection (i.e., collapse of cytokeratin matrix, G2 cell cycle arrest) (Doorbar *et al.*, 1991, 1997, 2006; Munger *et al.* 2002; Davy *et al.* 2005). The E1, E2, L1 and L2 ORFs are particularly well conserved among all members of the family. Most cis-responsive elements are in the long control region (LCR) between L1 and E6, a segment with little sequence conservation.

The direct link between papillomavirus infection and neoplasia, and the relationship between malignant progression and environmental or genetic cofactors was first established for animal papillomaviruses particularly cottontail rabbit papillomavirus (CRPV) (Kreider *et al.* 1985), bovine papillomavirus (BPV) (Jarrett *et al.* 1978; Campo 1992; Campo *et al.* 1994a), and canine oral papillomavirus (COPV) (Watrach 1969) (reviewed in Campo 2002).

The canine, cottontail rabbit and bovine papillomaviruses have been used as model systems in which to study the interaction of a papillomavirus with its natural host and with environmental cofactors (Jarrett *et al.* 1978; Campo *et al.* 1994b; Nicholls and Stanley 1999) and have been for many years the model system with which to study the biology of human papillomaviruses (HPV).

1.2.1 BOVINE PAPILLOMAVIRUSES

Bovine papillomaviruses fall into two subgroups (Jarrett *et al.* 1984): subgroup A and B. The subgroup A comprises the fibropapillomaviruses BPV-1, -2 (also known as delta-papillomavirus (Figure 1.5) (de Villiers *et al.* 2004) and BPV-5 (or epsilon-papillomavirus) (Figure 1.5) (de Villiers *et al.* 2004) that also causes cutaneous papillomas.

The subgroup B (or Xi-papillomavirus) (Figure 1.5) (de Villiers *et al.* 2004) comprises the epitheliotrophic papillomaviruses BPV-3, -4, -6.

The fibropapillomaviruses have a larger genome (approximately 7.9Kb) than the epithelial papillomaviruses, whose genome is approximately 7.3Kb.

In cattle BPV infection causes skin warts (BPV-1 and -2), papillomatosis and cancer of the upper gastrointestinal (GI) tract (BPV-4), papillomatosis of teats and udder (BPV-1, -5 and -6) and penis (BPV-1) and cancer of the urinary bladder (BPV-1 and -2) (Campo 1997). Cancer of the upper GI tract and urinary bladder develop as a result of the interactions between the virus, chemical carcinogens and immunosuppressant present in bracken fern (Campo 1997; Beniston *et al.* 2001; Beniston *et al.* 2003; Beniston *et al.* 2005).

Sequencing of the viral genomes has permitted molecular and genetic analysis, with type 1 and 4 being used as representatives for each group in the following discussion.

1.2.1.1 Bovine papillomavirus type 1

BPV-1 is a fibropapillomavirus that infects both epidermal keratinocytes and the underlying dermal fibroblast, resulting in the production of benign tumours.

Those papillomaviruses that are associated with fibropapillomas such as BPV-1 have the capacity to transform a variety of rodent cells in tissue culture. The transformation capacity of BPV in tissue culture was first demonstrated in the early 1960s (Black *et al.* 1963; Boiron *et al.* 1964; Thomas *et al.* 1964). In the early 1980s, quantitative focus assays were developed for BPV-1 using mouse and hamster cell culture (Dvoretzky *et al.* 1980). This transformation property has permitted investigators to study the viral functions involved in cellular transformation and in the induction of

cellular proliferation, even in the absence of a tissue culture system for the productive replication of the virus. Most of our knowledge of BPV-1 genetics has relied upon this capacity of BPV-1 to transform rodent cells, particularly mouse C127 cells (Lambert *et al.* 1988).

The DNA of BPV-1 was the first to be completely sequenced (Chen *et al.* 1982). Sequence analysis has identified the position of large open reading frames (ORFs) that revealed a genetic organization common to the human papillomavirus type 1a (HPV-1a) (Danos *et al.* 1982).

1.2.1.2 Bovine papillomavirus type-4

Bovine papillomavirus type 4 (BPV-4) infects the mucous epithelium of the alimentary canal of cattle and causes papillomas, which can progress to malignancy in animals that feed on bracken fern (Jarrett *et al.* 1978; Campo *et al.* 1994a). It has been shown that in naturally occurring bovine alimentary cancers the *ras* gene is rearranged and activated (McCaffery *et al.* 1989; Campo *et al.* 1990), p53 gene is mutated (Scobie *et al.* 1997) and the level of EGF receptors is increased (Smith 1987). These observations support the hypothesis that multiple independent events are necessary for the development of cancer. Early studies of the transforming properties of BPV-4 *in vitro* were conducted in established mouse fibroblast cells. The virus was found to transform both NIH3T3 and C127 established cells *in vitro* (Campo *et al.* 1983; Smith and Campo 1988). Work on C127 cell lines defined the BPV-4 E7 and BPV-4 E5 (described in further details in sections 1.2.4.5 and 1.2.5.2, respectively) as the transforming proteins of BPV-4 (Smith and Campo 1988). Later work looked at BPV-4 transformation of primary bovine fibroblasts (PalF) from the foetal palate (Jaggar *et*

al. 1990). BPV-4 is incapable of transforming PalF cells by itself and requires the cooperation of an activated *ras* gene to morphologically transform them. The partially transformed PalFs have an extended life span and are capable of anchorage-independent growth but are not immortal or tumorigenic in nude mice (Jaggar *et al.* 1990). In those cells it is the combination of BPV-4 E7, BPV-4 E5 and activated *ras* that induce cell transformation (O'Brien *et al.* 1999; Ashrafi *et al.* 2000) but immortalisation is conferred by HPV-16 E6 (Pennie *et al.* 1993), as the BPV-4 genome does not encode an E6 protein (Jackson *et al.* 1991).

BPV-4 shares many of the features of BPV-1, size and genome organisation are very similar (Patel *et al.* 1987), the only major difference is the substitution of the ORF encoding the transforming protein E6 with the ORF encoding the E5 oncoprotein (formerly known as E8) (Jackson *et al.* 1991).

1.2.2 HUMAN PAPILLOMAVIRUSES

The Human Papillomaviruses (HPVs) are divided into two main groups: those that infect cutaneous epithelia and produce skin warts, and those that infect mucosal epithelia, which give rise to laryngeal and anogenital warts. Of the more than 100 identified types of HPVs, approximately 30 specifically infect the genital epithelium (zur Hausen 2002). Recently all grouped in the Alpha-papillomavirus genus (de Villiers *et al.* 2004) these viruses are subdivided into low-risk (HPV-6 and HPV-11) as they induce lesions at low-risk for progression to malignancy and high-risk (HPV-16, HPV-18, HPV-31, HPV-33, HPV-45), associated at a high frequency with the development of malignant lesions. Approximately 99% of cervical cancers contain

HPV DNA of the high-risk types (Walboomers *et al.* 1999). The World Health Organization (WHO) in 1995 recognized HPV-16 (Human papillomavirus-type 16) and HPV-18 as *carcinogenic to humans* and HPV-31 and HPV-33 as *possibly carcinogenic to humans*. Later in February 2005 twenty-five scientists from 13 countries met to re-assess the carcinogenicity of HPVs. They concluded that HPV types 16,18,31,33,35,39,45,51,52,56,58,59,66 (grouped in the genus alpha) (de Villiers *et al.* 2004) (Figure 1.5) are *carcinogenic to humans* and HPV-6, -11 (genus alpha) and some HPV types of genus beta (including HPV-5 and HPV-8) are *possibly carcinogenic to humans* (IARC Monographs on the evaluation of carcinogenic risks to human 2005).

The Beta-papillomaviruses include some HPV types (among them HPV-5, HPV-8) associated with epidermodysplasia verruciformis (EV), a cutaneous neoplastic disease with a genetic component (Orth *et al.* 1978; Ramoz *et al.* 2002). In carriers who are not genetically predisposed, beta-, and the related gamma-papillomaviruses normally seem to establish asymptomatic infections or, at worst, induce small benign cutaneous lesions. However, it is thought that some EV HPV types viruses may be involved in the development of non-melanoma skin cancer (NMSC) also in the general population (Harwood *et al.* 2004).

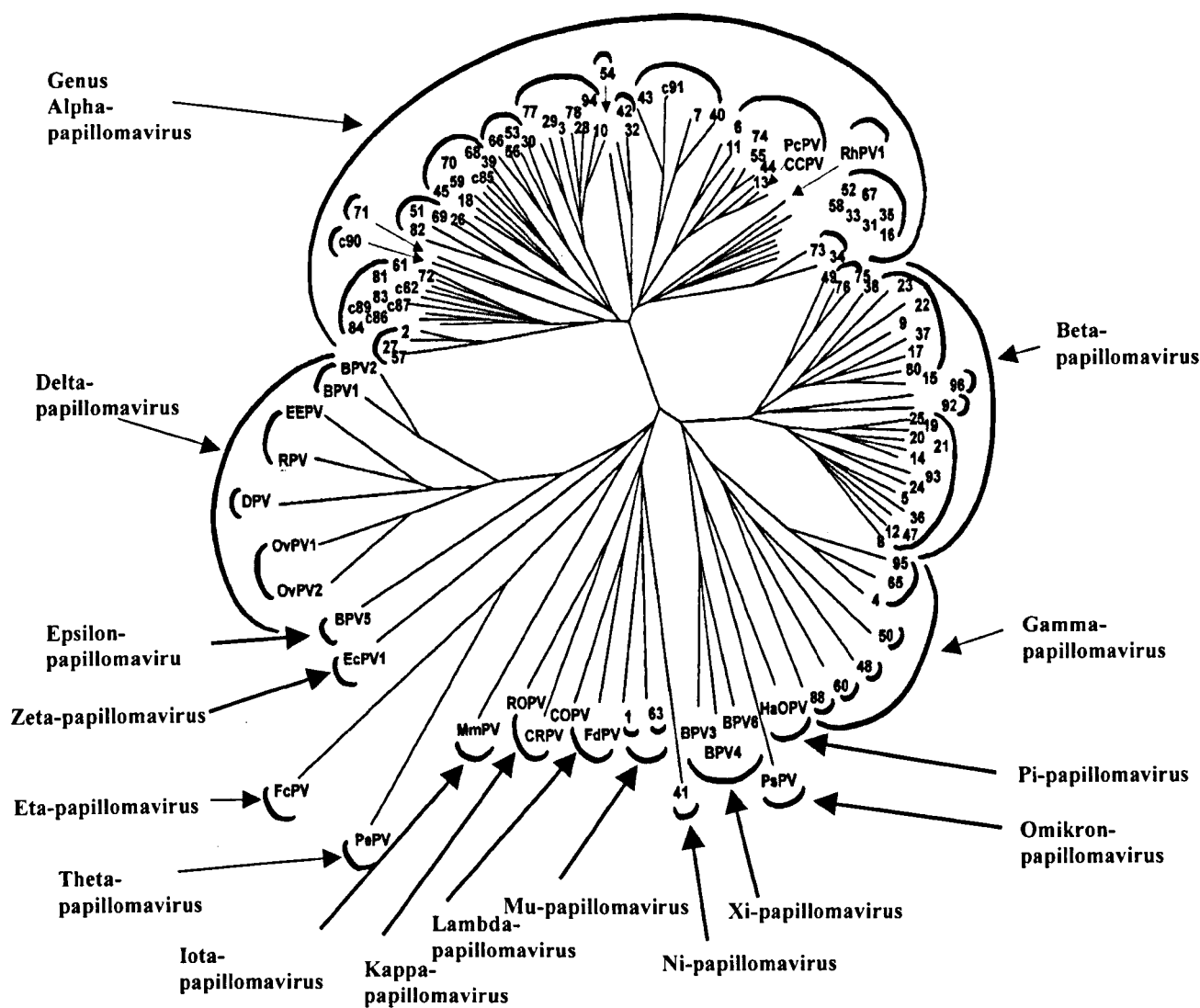


Figure 1.5 Phylogenetic tree containing the sequence of 118 papillomavirus types (Picture modified from de Villiers *et al.* 2004)

1.2.3 VIRUS AND CELL DIFFERENTIATION

The initial viral infection requires access of infectious particles to cells, presumably epithelial stem cells, in the basal layer of stratified epithelium, which for some HPV types is thought to require a microlesion at this site. Following infection and uncoating, it is thought that the virus maintains its genome as extrachromosomal elements in the nucleus of the basal cells of the epithelium and is maintained at around 10-200 copies per cell. The expression of viral proteins is regulated in line with cell differentiation so that viral genome replication is carried out in the cells of the spinous and granular layers, and capsid proteins are made only in the granular and squamous cells of the upper layers, and the mature virus is then released from the upper squamous layer. As infected cells divide, viral DNA is distributed between both daughter cells. One of the daughter cells migrates away from the basal layer and initiates a program of differentiation. The other daughter cell continues to divide in the basal layer and provides a reservoir of viral DNA for further cell divisions. Since production of papillomaviruses is restricted to suprabasal cells, the cells in the basal layer are not lysed by virion production but continue to proliferate. This differentiation-dependence allows the infected cells to persist in the basal layers for periods as long as several years (Figure 1.6). Reviewed in (Stubenrauch and Laimins 1999; Doorbar 2005).

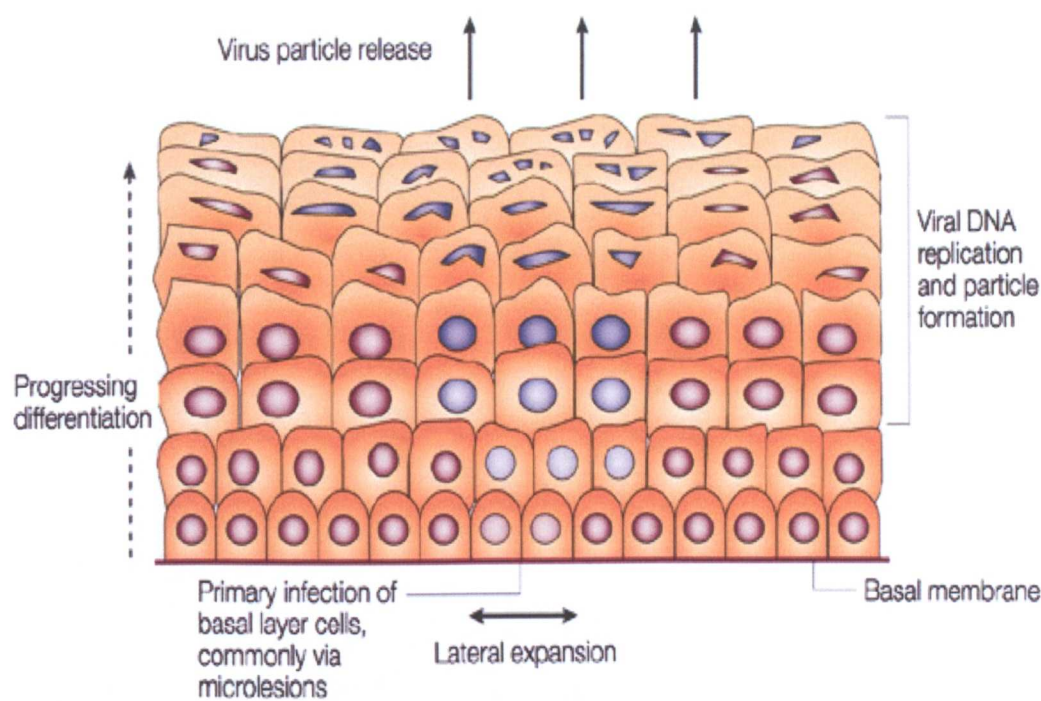


Figure 1.6 Human papillomavirus life-cycle. Papillomavirus generally enters the epithelium through micro-trauma and infects the basal layer cells. When a basal cell divides, a daughter cell will migrate into the suprabasal differentiating cell layers, where viral genes are activated in line with cell differentiation. Viral genome replication is carried out in the cells of the spinous and granular layers, and capsid proteins are made only in the granular and squamous cells of the upper layers, and the mature virus is then released from the upper squamous layer.

(Picture courtesy of Harald zur Hausen 2002, *Nature Reviews Cancer* 5, 342-350).

1.2.4 PAPILLOMAVIRUS GENOME ORGANISATION

The PVs genome can be divided into three regions, the early and late open reading frames separated by a long control region (LCR) or upstream regulatory region (URR). The early (E) proteins are those involved in cell transformation and viral replication whereas the two late (L) proteins are the capsid proteins for packaging the virus before release. The LCR or URR between late and early open reading frames contains the origin of replication, and regulatory elements that coordinate the expression of the viral proteins via proximal and distal promoters. In the viral LCRs are present multiple E2 DNA binding sites (BS) (12 in BPV-1, 4 in BPV-4 and 4 in HPV-16). E2 is the viral transcription regulator, and its binding to palindromic consensus sequence ACCGN₄CGGT, that differ in the sequences of the central N₄ “spacer nucleotides”, in the viral LCRs activates or represses transcription of the viral genes (described in details in section 1.2.4.2) (Romanczuk *et al.* 1990; Bouvard *et al.* 1994b). The majority of mRNA species are initiated at promoters inside or near to the LCR, with gene expression largely controlled post-transcriptionally (reviewed by Lambert *et al.* 1988; Longworth and Laimins 2004) (Figure 1.7).

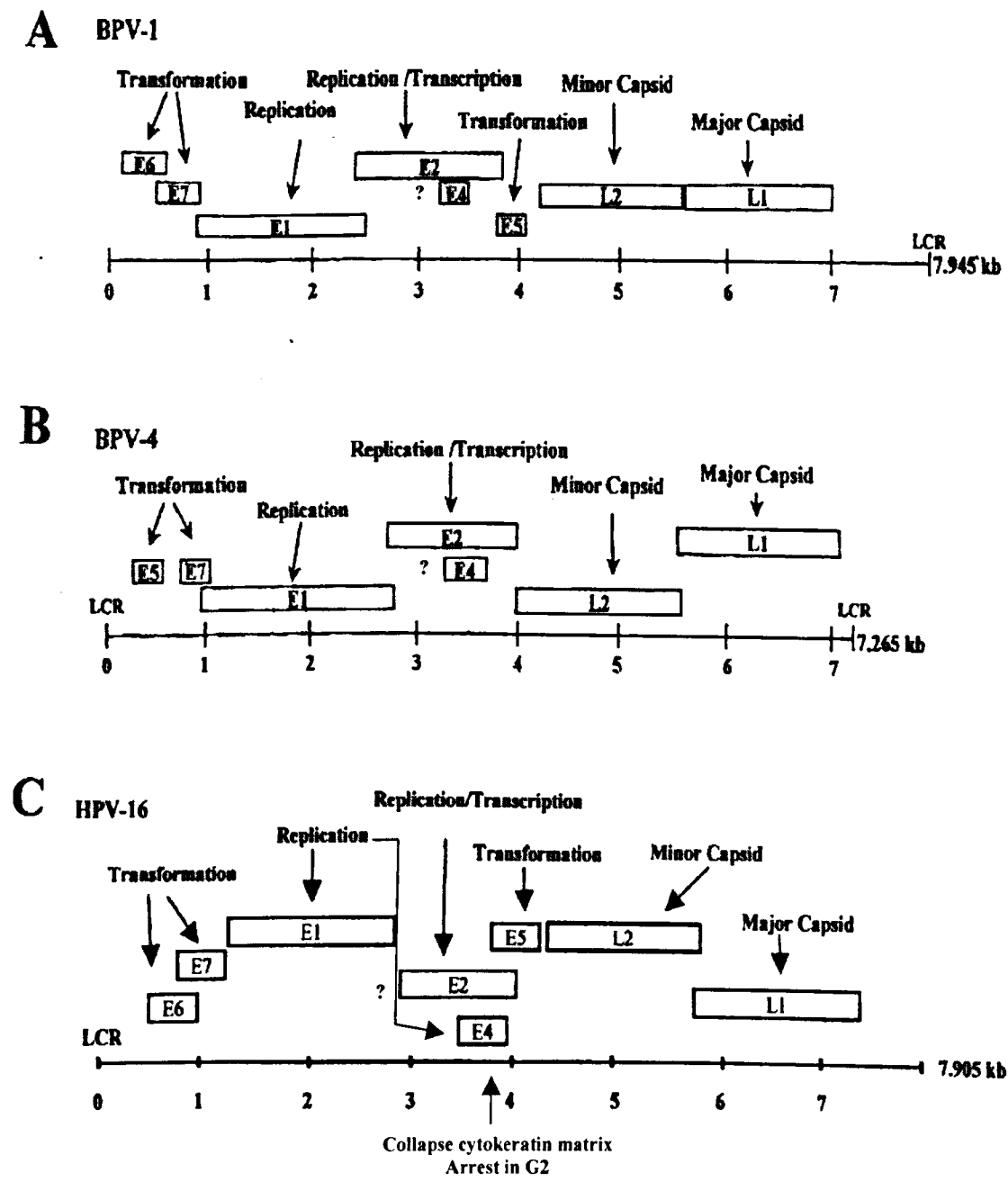


Figure 1.7 Diagrammatic genomic organisation of BPV-1, BPV-4 and HPV-16.

A, B, C) The viral genomes are represented as linear, with the open reading frames (ORF) as rectangles. The function of the proteins encoded by the individual ORF is indicated. (Picture courtesy of Papillomavirus Research: From Natural History to Vaccines and Beyond, 2006).

1.2.4.1 E1 ORF

E1 proteins, in conjunction with their cognate viral E2 proteins, are the origin recognition proteins for papillomaviruses (Ustav *et al.* 1991; Wilson and Ludes-Meyers 1991; Yang *et al.* 1991), recruit host cell replication proteins to the origin (Park *et al.* 1994; Bonne-Andrea *et al.* 1995; Conger *et al.* 1999; Han *et al.* 1999; Amin *et al.* 2000), and have intrinsic ATPase/helicase activity (Hughes and Romanos 1993; Yang *et al.* 1993; Seo *et al.* 1993) that functions in origin unwinding and fork progression (Gillette *et al.* 1994; Liu *et al.* 1995) reviewed by (Wilson *et al.* 2002).

1.2.4.2 E2 ORF

BPV 1 encodes three E2 proteins, the major transcription transactivator (E2TA), the product of the full-length E2 ORF, which activates viral promoters by binding to E2 responsive elements. The function of the E2TA is repressed by two other E2 proteins; the E2 transcriptional repressor (E2TR) encoded by the 3' half of the E2 ORF and E8/E2 transcriptional repressor (E8/E2TR), a translation product of a viral spliced RNA which fuses the upstream E8 ORF to the 3' end of the E2 ORF (Lambert *et al.* 1987; Choe *et al.* 1989; Lambert *et al.* 1990). The E2TA transactivator protein can be divided into three domains. The amino-terminal ~200 aminoacids comprise the transactivation domain of E2, and the carboxy-terminal ~100 aminoacids mediate DNA binding and dimerization. These domains are linked by a variable region called the hinge (Mc Bride *et al.* 1997). The transactivation domain is critical for the segregation of the viral genome to daughter nuclei, DNA replication, and

transcriptional regulatory functions of the E2 protein (McBride *et al.* 1989; Winokur *et al.* 1996; Skiadopoulos and McBride 1998; Bastien and McBride 2000). The segregation of viral genome requires the interaction of the amino-terminal transactivation domain of E2 with mitotic chromosomes (Bastien and McBride 2000). E2 interacts with a cellular bromodomain protein, Brd4, to mediate the segregation of viral genomes into daughter cells. Brd4 binds acetylated histones and has been observed to diffusely coat mitotic chromosomes in several cell types (McPhillips *et al.* 2005).

Mutation of just two amino acids, in the region of the transactivation domain that is crucial for the mitotic chromosome binding function of E2, eliminates chromosome binding activity but retains the ability to support DNA replication in cooperation with the viral E1 helicase (Baxter *et al.* 2005).

Also the E2 protein of BPV-4 is a transcription regulator, which acts on the BPV-4 long control region (LCR) to regulate transcription. There are four binding sites in the BPV-4 LCR, binding site 1 (BS1), 2 (BS2), 3 (BS3), and 4 (BS4) of which BS4 and BS2 mediate transactivation by E2, whereas BS1 and BS3 are responsible for repression by elevated levels of E2 (Jackson *et al.* 1995; Morgan *et al.* 1998). These data suggest that each of the four E2 sites performs different functions in the control of transcription and that competition between cellular transcription factors and viral E2 protein is essential in regulating the level of viral gene expression during papilloma development (Jackson and Campo 1995).

The DNA-binding domain of HPV-16 E2 has been shown to make functional interactions with the replication protein E1 and several proteins have been identified that interact with E2 proteins including TBP (Steger *et al.* 1995), AMF1 (Breiding *et al.* 1997), p300/CBP (Lee *et al.* 2000), SMN (Strasswimmer *et al.* 1999), TFIIB

(Rank and Lambert 1995; Benson *et al.* 1997), TopBP1, RACK1, POMP, p27^{BBP} (Boner and Morgan 2002) and several of these can modulate the transcription and replication properties of E2.

1.2.4.3 E4 ORF

Mutational analysis that prevented synthesis of the BPV-1 E4 ORF showed no effect on transcription, replication of viral genome, or cell transformation (Neary *et al.* 1987) confirming the observation of others (Doorbar *et al.* 1986) that the E4 open reading frame of papillomavirus is associated with late gene expression during papilloma formation. The E4 gene product is found predominantly in the cytoplasm of cells of the suprabasal epithelium (Doorbar *et al.* 1986; Chow *et al.* 1987; Crum *et al.* 1990).

The BPV-4 E4 ORF is transcribed into several RNA species (Stamps and Campo 1988). Two of these 7E11 and 1.6Kb transcripts, encode a potential E1^{E4} fusion protein, although a different region of E1 is found in these two transcripts. The 7E11 transcript is most likely to encode the E1^{E4} fusion peptide described for HPV-1a (Doorbar *et al.* 1988). The expression of BPV-4 E4 is greatest in the differentiated layers of papillomas (Anderson *et al.* 1997).

HPV encodes three late proteins, which are produced only in terminally differentiating keratinocytes, two of which L1 and L2 are structural components of the virion. The third, E1^{E4}, is derived primarily from the E4 ORF, which represents a region of maximal divergence between different HPV types. The expression of the HPV 16-E1^{E4} protein in human keratinocytes results in the total collapse of

cytokeratin matrix (Doorbar *et al.* 1991). Expression of the viral E1^{E4} protein is lost during malignant progression, but in premalignant lesions, E1^{E4} is abundant in cells supporting viral DNA amplification. Expression of 16-E1^{E4} in cell culture causes G2 cell cycle arrest using a novel mechanism in which it sequesters CdkI/cyclin B1 onto the cytokeratin network preventing their accumulation in the nucleus and hence prevents mitosis probably playing a role in creating an environment optimal for viral DNA replication (Davy *et al.* 2005).

1.2.4.4 E6 ORF

Since the complete sequencing of the BPV-1 DNA (Chen *et al.* 1982) further genetic dissection has identified E5 and E6 as the major transforming proteins of BPV-1 (Schiller *et al.* 1984; Yang *et al.* 1985; Neary and DiMaio 1989). Both E5 and E6 protein have been found in transformed cells (Androphy *et al.* 1985; Schlegel *et al.* 1986).

The 15.5kDa E6 protein is localised in both nuclear and membrane fractions of transformed cells (Androphy *et al.* 1985). (The E5 protein is described in further detail in section 1.2.5.1).

E6 it is capable of binding zinc through Cys-X-X-Cys motifs (Barbosa *et al.* 1989) and stimulates transcription when targeted to a promoter (Lamberti *et al.* 1990). It is capable of causing cell transformation *in vitro* (Androphy *et al.* 1985), but through a mechanism involving both transcriptional activation (Vousden *et al.* 1989; Chen *et al.* 1997; Ned *et al.* 1997) and binding to cytoskeletal and vesicular proteins (Tong *et al.* 1997; Tong *et al.* 1998). It does not bind or promote degradation of p53 (Chen *et al.*

1997). Furthermore, BPV-1 E6 sensitises mouse cells to tumour necrosis factor α (TNF)-induced apoptosis (Rapp *et al.* 1999). Viral protein may suppress or delay programmed cell death (PCD) (Lockshin *et al.* 1965) long enough for the production of virus progeny. On the other hand, induction of PCD at late stages of infection by viruses may represent a final and important step in spreading progeny to neighbouring cells. Regulation of PCD is important in terms of pathogenesis of diseases.

BPV-1 E6 also reduces surface Fas expression decreasing the transduction of apoptotic signals upon cross-linking with Fas ligand hence reducing Fas-mediated apoptosis (Liu *et al.* 2005).

An unusual feature of BPVs of subgroup B, such as BPV-4, is their lack of the E6 ORF which has apparently been replaced by the E5 ORF (Jackson *et al.* 1991) described in detail in section 1.2.5.2.

The HPV E6 ORF has been characterised most extensively, it encodes a 16-19kDa cell transforming protein. Its transforming ability was discovered by viral expression studies in human primary fibroblasts (Watanabe *et al.* 1989). In murine cells persistent E6 expression is capable of causing transformation, and in K14E6 transgenic mice, in which the HPV-16 E6 gene is directed in its expression by the human keratin 14 promoter (hK14) to the basal layer of the epidermis, E6 induced cellular hyperproliferation and epidermal hyperplasia and caused skin tumours in adult mice (Song *et al.* 1999). E6 alone is not capable of immortalising primary human foreskin keratinocytes (HFK) but can efficiently immortalise human mammary epithelial cells (MECs) (Kiyono *et al.* 1997; Liu *et al.* 1999). In high-risk HPVs such as HPV-16 however, E6 and E7 proteins are necessary and sufficient to immortalise human squamous epithelial cells (Watanabe *et al.* 1989; Hawley-Nelson *et al.* 1989; Munger *et al.* 1989).

The E6 protein is capable of binding zinc through four Cys-X-X-Cys motifs that form two zinc finger domains (Barbosa *et al.* 1989) and it may also act as a nucleic acid binding protein. In high-risk HPVs it has been shown to complex with p53 and to promote its degradation via the ubiquitin pathway (Scheffner *et al.* 1990), while low risk HPV E6 protein binds p53 less efficiently. p53 normally protects the cell from mutations by causing cell cycle arrest or apoptosis in the event of DNA damage. Chromosome damage has been found in E6-transformed cells (Coursen *et al.* 1997), and E6 expression has been shown to abrogate checkpoints in the cell cycle in a manner dependent upon p53 binding and degradation (Thompson *et al.* 1997). It has been shown to activate telomerase (Klingelutz *et al.* 1996) a chromosome repair enzyme that is typically upregulated in immortal cell lines. E6 protein has also been shown to interact with cellular minichromosome maintenance proteins which are believed to play a key role in regulating cellular DNA replication (Kuhne and Banks 1998; Zimmermann *et al.* 2000). In addition to interacting with p53, E6 may impact on apoptosis through p53-independent pathways. E6 can interact with and inhibit Bak, a proapoptotic protein expressed at high levels in the upper layers of differentiating epithelium (Thomas *et al.* 1998). The viral antiapoptotic function coupled with the growth promoting functions, can contribute to uncontrolled growth and oncogenesis. HPV E6 proteins target the PDZ-domain containing proteins such as the membrane-associated guanylate kinases (MGUKs) for proteasome-mediated degradation (Glaunsinger *et al.* 2000; Nakagawa *et al.* 2000). MAGUKs include the tumour suppressor proteins hDlg and hScrib, involved in controlling cell polarity and also the membrane associated guanylate kinase with inverted domain (MAGI-1) which is believed to be involved in the regulation of cellular signal transduction pathways. The

E6 proteins from high-risk HPVs differ in their ability to target these proteins in a manner that correlates with their malignant potential (Thomas *et al.* 2001).

In a study in K14E6 transgenic mice, in which a mutated HPV-16 E6 gene lacking the PDZ ligand motif was directed in its expression by human keratin 14 promoter (hK14) to the stratified squamous epithelia, it has been shown that the PDZ ligand domain of HPV-16 E6 is necessary for the induction of epithelial hyperplasia in mice (Nguyen *et al.* 2003) .

1.2.4.5 E7 ORF

Early studies in NIH3T3 cells established the product of the E7 ORF as the major transforming protein of HPV-16 (Vousden *et al.* 1988).

Three domains have been identified: a retinoblastoma protein (Rb) binding domain (Dyson *et al.* 1989), one zinc finger structure (Barbosa *et al.* 1989) and substrate sites for protein kinase C and casein kinase II (CKII) (Firzlaff *et al.* 1989; Armstrong and Roman 1995). The E7 protein blocks the negative regulation of retinoblastoma tumour suppressor protein by binding to hypophosphorylated Rb and Rb-related proteins and may also degrade Rb family members such as p107 and p130 (Boyer *et al.* 1996).

The E7 protein of high-risk HPV-16 and HPV-18 bind hypophosphorylated Rb with higher affinity than low-risk HPV-6 and HPV-11 (Griffiths and Mellon 1999).

Furthermore E7 has also been associated with AP-1 transcription factor (Antinore *et al.* 1996), the TATA box binding protein and members of the cyclin system (Tommasino *et al.* 1993; Massimi *et al.* 1997). These associations contribute to the ability of E7 to induce cellular proliferation, immortalization and transformation.

The E7 protein of BPV-1 contains neither Rb-binding domain or CKII sites and is consequently non-transforming. It has been reported that BPV-1 E7 contributes to the control of viral DNA replication and copy number of viral genomes (Lusky and Botchan 1985; Jareborg *et al.* 1992) rather than transformation per se.

A recent work correlates the lack of the canonical pRb-binding domain in the E7 ORF of artiodactyl papillomaviruses such as BPV-1, -2, -5, european elk PV (EEPV), deer PV (DPV), ovine PVs types 1 and 2 (OvPV-1 and -2) with the development of fibropapillomas. Nevertheless, only when the presence of the E5 protein is coupled to the absence of an E7 pRb-binding domain, does PV infection trigger the exclusive development of fibropapillomas suggesting a role for E5 in the pathogenic mechanism of fibropapilloma development (Narechania *et al.* 2004).

The BPV-4 E7 is one of the major transforming protein, capable of inducing morphological transformation of primary bovine fibroblast (PalF) cells in the presence of activated *ras* (Pennie *et al.* 1993). It is expressed in all layers of papillomas at all stages, consistent with its pivotal role in cell transformation. Its cellular localization however appears to change, being nuclear in the basal and suprabasal layers, and cytoplasmic in the more differentiated upper layers (Pennie *et al.* 1993; Anderson *et al.* 1997; Campo 1997). It possesses DNA-binding zinc finger and an Rb-binding domain but lacks the CKII site which is the main feature of the E7 protein of the high-risk HPVs (Jaggar *et al.* 1990).

1.2.4.6 L1 and L2 ORFs

These ORFs encode the two capsid proteins L1 and L2 that are expressed before the virus is shed from the squamous layer (Zhao *et al.* 1998). They have been useful for

the production of a BPV-4 vaccine (Chandrachud *et al.* 1995) and encode virus neutralizing epitopes that are effective prophylactic vaccines which prevent infection (Kirnbauer *et al.* 1996; Campo *et al.* 1997). The major capsid protein L1 has the intrinsic capacity to self-assembles into virus-like particles (VLP). When L2, which is not required for assembly, is coexpressed with L1, in eukaryotic cells by recombinant baculovirus or vaccinia virus, both L1 and L2 are incorporated into the particles (Hagensee *et al.* 1993; Kirnbauer *et al.* 1993).

Several groups have begun to assess VLP vaccines in clinical trials with encouraging results suggesting the possibility to develop effective prophylactic vaccines (reviewed in Galloway 2003).

The L1 ORF encodes the 56-60 kDa major capsid protein, the minor capsid protein L2 has a molecular weight of 52-58 kDa. The L1 ORF is the most conserved gene within the PVs genome and has therefore been used for the identification of new PV types over the past 15 years (de Villiers *et al.* 2004). Also the N-terminal domain of the L2 proteins is highly conserved among different papillomaviruses. Differentiation of the epithelium triggers a coordinate increase in the replication of the viral genome and expression of the L1 and L2 structural viral proteins leading to the assembly of infectious viral particles in the nucleus.

The L2 protein is believed to be involved in encapsidation of the viral DNA, generation and infectivity of PV virions as well as interaction with cell surface receptors (Kawana *et al.* 2001; Okun *et al.* 2001). The L2 protein of HPV 16 contain nuclear localisation signal (NLS), can interact with several cellular host proteins, and has the ability to recruit them to the nucleus (Gornemann *et al.* 2002). It has been shown that HPV-16 L2 selectively inhibits the transcriptional activation property of E2 and that there is a direct interaction between the two proteins, although this is not

sufficient to mediate the transcriptional repression (Okoye *et al.* 2005). The HPV-16 L2 has also been shown to bind β -actin (Yang *et al.* 2003) causing cell retraction and disruption of the microfilament network possibly assisting in the release of mature virus.

1.2.5 The E5 proteins of Papillomaviruses: common and different features

All the PVs share a similar genome organisation and encodes at least one transforming protein. They also possess an early open reading frame that encode for a small hydrophobic transforming protein, the E5. Whatever the function of E5 *in vivo*, its role *in vitro* cell transformation is being rapidly elucidated and it has become clear that despite its small size, the protein has wide pleiotropic effects. Here it is reviewed what is known about the products of the E5 open reading frames from BPV-1, BPV-4 and HPV-16.

1.2.5.1 BPV-1 E5

E5 is the major transforming protein of BPV-1. The E5 ORF encodes a 44 amino acids long highly hydrophobic protein that contains two distinct domains: an amino terminal hydrophobic region that spans the cell membrane and a 14 amino acids hydrophilic carboxy terminal domain, which contains two cysteine residues that mediate homodimer formation via disulfide bonds (Schlegel *et al.* 1986; Horwitz *et al.* 1988; Burkhardt *et al.* 1989; Surti *et al.* 1998). Although E6 is also involved in cell transformation (section 1.2.4.4) mutational analysis of E5 showed that it is essential

for cell transformation (DiMaio *et al.* 1986). It is capable of transforming a number of different cell lines (Schiller *et al.* 1986; Bergman *et al.* 1988; Leptak *et al.* 1991). The protein is localised largely to the membranes of the endoplasmic reticulum and Golgi apparatus of transformed cells with its carboxy-terminus oriented intraluminally (Burkhardt *et al.* 1989). It has been shown to bind and activate the β receptor for platelet derived growth factor (PDGF β -R) (Petti *et al.* 1991; Petti and DiMaio 1992; Goldstein *et al.* 1994), but it does not directly activate other receptors, including the closely related PDGF α receptor or the epidermal growth factor (EGF) receptor (Goldstein *et al.* 1994; Petti *et al.* 1994; DiMaio and Mattoon 2001). This is consistent with the tissue tropism of the BPVs capable of infecting fibroblasts and other mesenchymal cells that express abundant PDGF β receptor. On the basis of extensive mutational analysis it has been proposed that BPV1-E5/PDGF β receptor complex formation requires an electrostatic bond between a juxtamembrane lysine in the PDGF β receptor and aspartic acid 33 of the BPV-1 E5 protein and an hydrogen-bond between a transmembrane threonine in the receptor and glutamine 17 of the BPV-1 E5 protein (DiMaio and Mattoon 2001). Moreover, mutational studies also indicate that glutamine 17 and aspartic acid 33 are important in transformation (Meyer A.N. *et al.* 1994; Sparkowski *et al.* 1996). The formation of the complex with PDGF β -R also requires dimerization of the E5 protein (Nilson *et al.* 1995; Mattoon *et al.* 2001), and glutamine 17 plays a role in stabilizing the E5 dimer which is oriented in an antiparallel fashion to PDGF β -R (Kulke *et al.* 1992; Klein *et al.* 1999; DiMaio and Mattoon 2001). After alanine scanning mutations analysis in the BPV1-E5 protein it has been proposed that each E5 monomer had an independent binding site for the PDGF β receptor, but the binding site was accessible only following E5 dimerisation (Adduci *et al.* 1999). On the basis of mutational analysis, molecular modeling,

analysis of the chimeric E5 proteins, and NMR studies (Surti *et al.* 1998; Klein *et al.* 1998; Klein *et al.* 1999; Mattoon *et al.* 2001) Di Maio and Mattoon (DiMaio and Mattoon 2001) proposed that each PDGF β receptor molecule interacts with the glutamine on one E5 monomer and the aspartic acid on the other. Thus, the E5 protein has to dimerise in order to generate intact binding sites for the PDGF β receptor, a model consistent with the finding that dimerisation-defective E5 mutants are also defective for inducing receptor activation and transformation.

1-E5 can activate PI-3-K (phosphoinositide 3-Kinase) and c-src independently of growth factor receptor activation (Suprynowicz *et al.* 2000; Suprynowicz *et al.* 2002). The BPV-1 E5 protein also binds to the 16 kDa transmembrane subunit c (also known as ductin) of the vacuolar proton pump H^+ -ATPase (V-ATPase) (Goldstein *et al.* 1991; Finbow *et al.* 1991; Goldstein *et al.* 1992b). This pump is responsible for controlling the pH of the Golgi apparatus and other intracellular organelles. 16K consists predominantly of 4 transmembrane (TM) domains. The association between the ATPase subunit and the E5 protein appears to be mediated largely by transmembrane interactions with the glutamic acid in position 143 of the 16K TM4 domain and the glutamine in position 17 within E5 as important contributors (Goldstein *et al.* 1992a; Andresson *et al.* 1995). Acidification of the Golgi apparatus is impaired in cells transformed by the E5 protein and it has been proposed that the E5 protein directly inhibits V-ATPase activity (Schapiro *et al.* 2000; Suprynowicz *et al.* 2000). Because many important growth regulatory proteins, including PDGF β receptor, pass through the Golgi apparatus en route to their final destination in the cell, the ability of the BPV E5 protein to perturb the pH of intracellular organelles may influence the activity of these proteins and contribute to transformation (DiMaio and Mattoon 2001).

1.2.5.2 BPV-4 E5

The E5 (formerly E8) open reading frame of BPV-4 has been found where the E6 ORF would be expected to be, and encodes a 42 amino acids long hydrophobic protein with homology to the BPV-1 E5 protein (Jackson *et al.* 1991). It is composed of two domains: a very hydrophobic region, with an α -helix transmembrane span, encompassing the first 30 residues of the protein, and a second region of mainly hydrophilic amino acids comprising the C-terminal 12 residues. It is localised in the endoplasmic reticulum, the Golgi apparatus and occasionally the plasma membrane (Pennie *et al.* 1993). *In vivo*, it is found in the basal, suprabasal and in differentiated cell layers of papillomas (Anderson *et al.* 1997; Araibi *et al.* 2004) and there are no data to suggest that it binds to or activates growth factor receptors.

Domain swap analysis between the BPV-1 E5 and BPV-4 E5 proteins showed that the C-terminal domain of 4-E5 is implicated in transformation and acts in a different way from that of BPV-1 E5 (O'Brien *et al.* 1999). In fact, the two C-terminal sequences of 1-E5 and 4-E5 show poor homology. It is not known whether the 4-E5 protein binds or not growth factor receptors. Growth factors activate a series of signal-transduction cascades, among which activation of the mitogen-activated protein kinase (MAPK) cascade, Ras-Raf-MEK-ERK, is the best characterized (Schaeffer *et al.* 1999). Most studies indicate that these signals eventually control G1 phase progression by regulating cyclin D1 expression, cyclin E and cyclin A synthesis (Pages *et al.* 1993; Weber *et al.* 1997; Balmain *et al.* 1999). Unlike the 1-E5 protein, 4-E5 upregulates cyclin A and cyclin A-associated kinases and a strong relationship exists between cyclin A promoter transactivation and cell growth and transformation (O'Brien and Campo 1998; O'Brien *et al.* 2001). In recent work it has been found that 4-E5-

mediated upregulation of cyclins is largely independent of the MAPK cascade and is probably also independent of growth factors (Zago *et al.* 2004).

Given the key role played by cyclin A-associated kinases in DNA replication, it is not surprising that DNA tumour viruses encode protein(s) capable of activating cyclin A transcription and inducing cyclin A-CDK activity (Ohkubo *et al.* 1994; Zerbass *et al.* 1995; Chang *et al.* 1997; Schulze *et al.* 1998). Recent data show that 4-E5 also acts to bypass repression at the CCRE/CDE (cell cycle repressor element or cell cycle-dependent element)(Grindlay *et al.* 2005). CCRE is one of the sites in the cyclin A promoter which is crucially involved in repression of transcription in quiescent cells and cells in early-G1 and is occupied only when cyclin A transcription is repressed (Blanchard 2000). E5 also transactivates the cyclin A promoter through the CCAAT box element in a NF-Y-dependent manner. NF-Y belongs to a family of CCAAT box-binding proteins involved in the control of the expression of many eukaryotic genes and it is believed that E5 transactivates the cyclin A promoter through NF-Y association with a p110 CCAAT-box binding factor (p110 CBF) (Grindlay *et al.* 2005).

The E5 protein of BPV-4 bind *in vitro* to the 16K protein causing loss of gap junction intercellular communication in PalFs (Faccini *et al.* 1996).

The evidence of a similar mechanism for cell transformation brought about by 1-E5 and 4-E5 comes from the fact that they both bind to 16K and inhibit gap junction intercellular communication (Faccini *et al.* 1996; Ashrafi *et al.* 2000). A number of mutational studies on BPV-4 E5 (Ashrafi *et al.* 2000) and HPV-16 E5 (Rodriguez *et al.* 2000; Adam *et al.* 2000) show that the ability of E5 proteins to bind 16K does not correlate with their transforming activity. Binding to 16K may provide an export mechanism for E5 transforming proteins to the Golgi and post Golgi compartments.

Once in the Golgi when the V-ATPase is first activated, E5 proteins may become dissociated from the 16K and bind to other targets such as growth factor receptors (Ashby *et al.* 2001).

1.2.5.3 HPV-16 E5

The HPV-16 E5 protein is 83 amino acids in length. Like the bovine papillomaviral 1-E5 and 4-E5 proteins, the 16-E5 and the others HPV E5 proteins are highly hydrophobic with conserved cysteines in the C-terminal domain (Bubb *et al.* 1988). However, they possess an additional transmembrane domain and lack the active group found at position 17 of BPV-1 E5 and BPV-4 E5 (Bubb *et al.* 1988). The E5 proteins of HPVs show only a weak transforming activity. HPV-16 E5 does not transform cells to the same extent as E6 and E7 but enhances the immortalization potential of E6 and E7 (Stoppler *et al.* 1996) and in cooperation with E7 stimulates the proliferation of human and mouse primary cells (Bouvard *et al.* 1994a; Valle and Banks 1995).

HPV-16 E5 has been shown to bind to a number of growth factor receptors (Hwang *et al.* 1995) such as EGF-R and PDGF-R, like BPV-1 E5, and also the colony stimulating factor-1 (CSF-1) (Nagata *et al.* 1993). However it seems that only the low risk type HPV-6 E5 could interact with the PDGF-R (Conrad *et al.* 1994). Studies monitoring the mitogenic response to EGF in E5-expressing human keratinocytes led investigators to propose a synergistic interplay between E5 and EGF (Straight *et al.* 1993). In a recent work in K14E5 transgenic mice, in which the HPV-16 E5 gene was directed in its expression by human keratin 14 promoter (hK14) to the basal layer of the epidermis, E5 alters the growth and differentiation of stratified epithelia and

induces epithelial tumors at a high frequency via a pathway requiring functional EGF-R (Genther Williams *et al.* 2005).

HPV-16 E5 also binds to 16 kDa ductin (Conrad *et al.* 1993) and this binding has been shown not only to inhibit gap junction communication but also endosome acidification and EGF-R degradation (Straight *et al.* 1995).

1.3 IMMUNE EVASION BY VIRUSES

Generally pathogens have a short generation time and the host defence must be proper, rapid and work at several levels. The first line of defence is represented by the skin and epithelia that can keep out many intruders, but viruses often possess specialised mechanism to overcome epithelial barriers. The next layer of defence is the rapid production of specialised cells and molecules of the innate immune system and these defences too can be penetrated. Finally, in many cases, lasting protection is afforded through acquired immunity. However, the persistence and repeated reactivation of many viruses must be enabled by specific evasion of adaptive immunity.

1.3.1 EVASION OF ADAPTIVE IMMUNITY

The MHC I molecules, which are essential for presentation of foreign peptides to the cytotoxic T lymphocytes (CTL), are targets of many pathogens, including viruses (Ploegh 1998; Tortorella *et al.* 2000). CTL recognize virus-infected cells through the specific interaction of their T-cell receptor with an MHC I molecule presenting a viral peptide. The MHC I complex (described in detail in section 1.1.3) consists of an heavy chain containing the peptide binding site and β_2 -microglobulin, which assemble very rapidly in the lumen of the endoplasmic reticulum (ER). Peptides, generated by the proteasome in the cytoplasm, are translocated by TAP (transporter associated with antigen processing) into the ER where they assemble in ternary complexes and are transported to the cell surface for presentation to CTL (York and Rock 1996). Once on the cell surface, some MHC I molecules are endocytosed and either recycled back

to the plasma membrane or retained intracellularly and degraded (Reid and Watts 1990; Abdel Motal *et al.* 1993) (the antigen processing and presentation is described in detail in section 1.1.3.1). Hence, maintenance of steady-state levels of functional MHC I complexes at the cell surface is achieved by a balance of several processes: the synthesis and exocytic transport of newly synthesized MHC I molecules, the recycling of endocytosed peptide-associated MHC I molecules, and the degradation of denaturated MHC I molecules that have lost antigenic peptides (Zuniga *et al.* 1999). Interference with the assembly and/or trafficking of the MHC I complex can contribute to the persistence of a virus. Several viruses that induce chronic infections encode proteins that target or modulate the host's immune system and have evolved strategies for evading CTL-mediated immunity by interfering with the cell surface display of MHC I (Ploegh 1998; Tortorella *et al.* 2000; Johnson *et al.* 2001).

1.3.1.1 Interference with the generation of antigenic peptide

During an infection, viral gene products expressed in the cytosol may be targeted for degradation and presented by MHC class I molecules to CTL that can act early to eliminate the infected cells (Spriggs 1996). Thus it is not surprising that several viruses have evolved a mechanism to evade the proteolysis and the subsequent generation of antigenic peptides.

Epstein-Barr virus (EBV) is a member of the γ -human herpesvirus family; it maintains a life-long latent association with B lymphocytes and a permissive association with stratified epithelium in the oropharynx (Greenspan *et al.* 1985; Yao *et al.* 1989). The EBV nuclear antigen-1 (EBNA-1) is produced early in the infected B

cell and in general CTLs specific for EBNA-1 are generated during T-cell development but fail to be activated during EBV infection suggesting that EBNA-1 peptides are not presented by class I molecules (Khanna *et al.* 1995). In fact EBNA-1 includes a unique glycine-alanine repeat (GAR) domain that inhibits the endogenous presentation of CTL epitopes through the class I pathway by blocking proteasome-dependent degradation of this antigen (Tellam *et al.* 2001).

Human cytomegalovirus (HCMV) is a large β -herpesvirus coding for more than 200 potential proteins (Chee *et al.* 1990). Primary infections by HCMV are followed by life-long infections that are asymptomatic in immunocompetent individuals, but lead to severe, even life-threatening, disease if the immune system is compromised. HCMV establishes latency in several somatic cell types, such as the monocytic and endothelial cells, from which reactivation occurs (Chee *et al.* 1990; Soderberg-Naucler *et al.* 1997; Fish *et al.* 1998). Ultimately, acute infections are controlled by CD8T cell. In order to evade this cytotoxic T-lymphocyte response HCMV contains several genes in its genome dedicated to interfere with the MHC class I pathway. In the immediate early phase of HCMV infection a CTL response is directed against antigenic peptides derived from a 72 kDa transcription factor. This CTL response is abrogated when the 72 kDa protein is expressed together with a protein that shows kinase activity, the matrix protein phosphoprotein 65 (pp65). The pp65 probably phosphorylates the 72kDa protein and inhibits the generation of its derived antigenic peptides. The phosphorylation might affect the 72kDa protein cleavage pattern or result in its failure to be appropriately ubiquitin-conjugated (Gilbert *et al.* 1996).

Human immunodeficiency virus (HIV) and influenza virus can modify antigenic peptides through random mutations. An antigenic peptide derived from HIV can even act as an antagonist peptide and thus prevent full activation of specific T cell (Klenerman *et al.* 1994; Bertoletti *et al.* 1994).

It has been shown that also hepatitis B virus (HBV) isolates derived from two chronically infected patients display variant epitopes that act as natural T- cell antigen receptor (TCR) antagonists with the capacity to inhibit the CTL response to the wild-type epitope (Bertoletti *et al.* 1994).

1.3.1.2 Interference with the antigenic peptide transport via the TAP (transporter associated with antigen processing) complex

The α -herpesviruses, herpes simplex virus type 1 (HSV-1) and type-2 (HSV-2) establish life-long infections with latent phases and recurrent acute infections leading to typical lesions (Roizman and Sears 1996). During latency, HSV remains in neurons which express very low levels of MHC class I molecules and do not seem to express proteins and this condition might prevent immune detection. Upon reactivation, however HSV replicates in MHC class I-containing epithelial cells, thus exposing the virus to T-cell surveillance. The HSV genome contains approximately 100 ORFs the products of which are potential targets for the CD8T cells. To escape or delay T-cell recognition, one of the immediate early proteins of HSV, the infected-cell protein 47 (ICP47), is dedicated to evasion of MHC class I antigen presentation. ICP47 of HSV-1 and HSV-2 inhibits peptide transport by preventing peptide binding by interacting with both TAP1 and TAP2 on the cytosolic side of the ER (York *et al.*

1994; Hill *et al.* 1995; Fruh *et al.* 1995). As consequence, empty major histocompatibility complex class I molecules are retained in the endoplasmic reticulum and recognition of HSV-infected cells by cytotoxic T lymphocytes is abolished (Galocha *et al.* 1997).

The US6 gene of HCMV encodes for a 21 kDa type I membrane glycoprotein that inhibits TAP function. This protein unlike ICP47, interacts with TAP complex on the luminal side of the endoplasmic reticulum. The interaction of US6 with TAP does not prevent peptide or ATP binding hence this interaction may prevent peptide transport by occluding the exit pore of the TAP complex (Hengel *et al.* 1996; Hengel *et al.* 1997; Ahn *et al.* 1997; Lehner *et al.* 1997).

HPV 11 and HPV 6 cause recurrent respiratory papillomas, benign tumours of the larynx and trachea (Gissmann *et al.* 1982). The E7 proteins encoded by these viruses are believed to contribute to the apparent loss of TAP1 function found in papilloma cells. If TAP1 is lacking or its function is abrogated there is a significant reduction in cell surface MHC I molecules bearing an antigenic peptide at the cell surface. It has been shown that E7 binds TAP1 and can inhibit ATP-dependent peptide transport resulting in poor presentation of viral antigen on HPV-infected cells (Vambutas *et al.* 2001).

1.3.1.3 Inhibition of MHC class I surface expression

The prototype for a viral protein interfering with MHC class I antigen presentation was discovered in non-oncogenic adenoviruses: the E19 protein encoded in the E3

region (Andersson *et al.* 1985; Burgert *et al.* 1985). This glycoprotein inhibits the intracellular transport of MHC class I molecules by forming stable complex in the ER (Cox *et al.* 1991). Such ER retention by E19 is attributed to a double lysine motif within its cytoplasmic tail. This dilysine motif interacts with coat protein-I (COP-I), involved in vesicles retrieval between the ER and Golgi, and is a common ER retention motif (Paabo *et al.* 1987; Jackson *et al.* 1990; Cosson and Letourneur 1994). E19 or a truncated version that lacks the ER retention signal can also bind to TAP, independently of its association with MHC class I molecules and the TAP-associated glycoprotein, tapasin (described in section 1.1.3.1). E19 may causes a decrease in class I/TAP association therefore delaying peptide loading of the MHC class I molecules (Bennett *et al.* 1999).

HCMV in the immediate early phase of virus infection expresses US3, a 23 kDa glycoprotein that retains class I molecules in the ER (Ahn *et al.* 1996). US3 interacts with peptide-loaded MHC class I molecules, but its mechanism of ER retention is not clear. HCMV also encodes two early gene products, US2 and US11, that selectively target class I heavy chains (HC) for degradation by the proteasome. In cells that express US2 or US11, class I HC are dislocated from the ER through the translocon (sec61p complex) and into the cytosol (Wiertz *et al.* 1996). It seems that class I HC are ubiquitinated largely after extraction from the ER membrane. The mechanism of degradation of class I HC by US2 and US11 is very similar to the degradation of misfolded and abnormal proteins in the ER (Plempner *et al.* 1999). Given the different mechanism of evasion shown by US3 and US2/US11 HCMV has developed different T-cell escape strategies at different times during the infectious cycle.

The mouse cytomegalovirus (MCMV) gene *m152* encodes a 40 kDa type I glycoprotein expressed early during infection that retains class I molecules within the ER-Golgi intermediate compartment, thus preventing surface expression of class I molecules (Ziegler *et al.* 1997).

The MCMV gene *m06* encodes a 48 kDa glycoprotein that forms a tight complex with β_2m -associated MHC I class I molecules. After passing the Golgi, the complex enters the endocytic route and reaches the lysosomes where both the viral protein and MHC class I molecule undergo rapid proteolysis (Reusch *et al.* 1999). The *m04* gene encodes a 34 kDa glycoprotein (gp34) that interacts with class I molecules within the ER and the complex is not retained but transported to the cell surface. Probably gp34 counteracts class I retention, perhaps to decrease susceptibility of infected cells to recognition by natural killer cells (Kleijnen *et al.* 1997).

HIV expresses two proteins that downregulate the expression of surface MHC class I molecules, Nef and Vpu (Piguet *et al.* 1999).

Vpu is an 81-residue oligomeric type I-anchored membrane protein that prevents the cell surface expression of class I molecules acting on newly synthesised class I molecules and inducing their destabilisation (Kerkau *et al.* 1997).

Nef is a 27-34 kDa multifunctional protein that has no apparent enzymatic activity and is thought to function by acting as an adaptor protein (AP). Nef binds to the cytoplasmic tail domain of class I HC (Harris and Neil 1994; Greenway *et al.* 1995; Grzesiek *et al.* 1996; Rossi *et al.* 1996; Schaefer *et al.* 2000; Williams *et al.* 2002), and contains a number of potential protein-protein interaction domains that are required for MHC I downmodulation.

Nef localises to the plasma membrane and accelerates endocytosis of class I complex in macrophages. The class I/Nef complex is then targeted to the lysosomes. Nef targets the HLA-A and -B locus products, but not the -C and -E locus products for lysosomal degradation (Le Gall *et al.* 1998; Cohen *et al.* 1999). This selective downmodulation results from amino acid sequence variation in the Nef-binding domain within the cytoplasmic tails of these molecules (Williams *et al.* 2002).

Nef disrupts MHC-I cell surface expression by a different mechanism from the one used to downmodulate CD4. In HIV-infected lymphocytes and astrocytic cells, the primary effect of Nef on MHC I is to disrupt its transport to the cell surface rather than to promote its endocytosis (Swann *et al.* 2001; Kasper *et al.* 2003). It has been found that Nef redirects MHC I from the trans-Golgi network (TGN) to lysosomes. In this process the $\mu 1$ subunit of adaptor protein, AP-1, which is required for proper sorting of lysosomal hydrolases from the TGN to lysosomes, is required for Nef to disrupt MHC I trafficking. Nef stabilises an interaction between MHC I and AP-1 and this interaction requires sequences from the N-terminal α helix of Nef and from the MHC I cytoplasmic tail (Kasper *et al.* 2005; Williams *et al.* 2005).

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia/lymphoma (ATLL) (Poiesz *et al.* 1980; Hinuma *et al.* 1981; Yoshida *et al.* 1982), as well as the neurologic disorder tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP-HAM) (Gessain *et al.* 1985; Rodgers-Johnson *et al.* 1985; Osame *et al.* 1986). HTLV-1 induces a lifelong chronic infection, which may result in ATLL in 1 to 5% of carriers 20 to 30 years after infection. The HTLV-1-encoded protein, p12^l, is a small hydrophobic protein that reside in the endoplasmic reticulum and Golgi apparatus and can physically bind the free human major

histocompatibility complex class I HC causing its intracytoplasmic retention and proteosomal degradation (Johnson *et al.* 2001).

1.3.2 EVASION OF INNATE IMMUNITY

1.3.2.1 Interferon and innate immune response

Interferons (IFNs) are the first cytokines to be molecularly characterized and have been extensively studied in the context of host defence against viral infection. IFNs regulate cellular antiviral, antitumour and immunological responses through IFN-stimulated gene expression.

There are two types of IFNs, type I IFNs (IFN- α and IFN- β) and type II IFN (IFN- γ). The IFN α/β are produced by a variety of cells upon viral infection, while IFN- γ is not virus inducible and is produced by activated T lymphocytes and natural killer cells (Lengyel 1982; Weissmann and Weber 1986; Pestka *et al.* 1987; Vilcek and Sen 1996). The IFNs α , β and IFN γ -inducible gene promoters are characterised by the presence of consensus elements known as IFN-stimulated response elements (ISRE) and IFN- γ activation sites (GAS), which mediate primary transcriptional induction in response to IFN- α/β and IFN- γ , respectively (Darnell *et al.* 1994).

Type I IFN production is stimulated early in the course of viral infection, and IFN production is an important determinant of the course of the subsequent disease (DeMaeyer 1988; Muller *et al.* 1994). IFNs act directly on the virally infected cell by interfering with viral replication and by inhibiting cellular proliferation. Immunomodulatory activities of IFNs also contribute to their antiviral roles. IFN-

γ enhances the expression of cellular proteins such as MHC class I molecules that contribute to immune-mediated lysis of virus-infected cells (DeMaeyer 1988).

The interferon regulatory factors (IRFs) constitute a family of transcription factors that are believed to play a critical role in the regulated expression of the IFN- α and IFN- β genes.

IRF-1 in particular can activate transcription from the ISRE, which is present in the promoters of gene activated by type I IFN and is itself IFN- γ inducible. Some members of the IRF family are involved in a variety of cellular growth control mechanisms. Seven human members of the family have been characterized: IRF-1, -2, -3, -4, -7, IFN consensus sequence binding protein (ICSBP) and interferon stimulated gene factor 3 (ISGF3 γ).

IRF-1 has been characterized as a transcriptional activator and an anti-oncogene whose functional loss contributes to aberrant growth (Fujita *et al.* 1989; Harada *et al.* 1990; Reis *et al.* 1992; Harada *et al.* 1993).

IRF-3 is part of a virus activated transcription factor complex and its transcriptional activity is increased in response to viral infection (Fujita *et al.* 1989; Schafer *et al.* 1998; Wathelet *et al.* 1998; Weaver *et al.* 1998).

The E6 protein encoded by HPV-16 can bind to IRF-3 and this interaction is specific and results in a marked reduction of the IRF-3 transactivation function *in vivo* (Ronco *et al.* 1998). The interaction of HPV-16 E6 with IRF-3 and the inhibition of its transcriptional activity could contribute to the ability of the virus to disrupt the cellular antiviral response.

The E7 proteins encoded by HPV-11, HPV-16 and HPV-18 physically interact with IRF-1 and abrogate its transactivation function, probably through histone deacetylation (Park *et al.* 2000; Um *et al.* 2002).

The functional inactivation of IRF-1 and IRF-3 by HPVs E7 and E6 proteins, respectively, could play an important role in the escape of immune surveillance of virus-infected cells. In fact this interaction could interfere with the transcriptional activation of some genes, such as genes involved in antigen presentation (TAP1), and possibly genes involved in the attraction of T lymphocytes to target cells (MCP-1) so leading to HPVs immune evasion.

1.3.2.2 Viral evasion of natural killer cells

Natural killer (NK) cells are a central component of the innate immune system (described in detail in sections 1.1.1 and 1.1.4) and are crucial in defence against certain viruses.

NK cells are activated during a wide variety of viral infections by virus-induced type I IFNs (Biron *et al.* 1999).

As described before in sections 1.3.1.1, 1.3.1.2 and 1.3.1.3, many pathogens have effective means of avoiding the adaptive immune response. In eluding T cells, however, these viruses might have increased their susceptibility to NK cell-mediated defences. Many viruses have developed mechanism to evade the NK cell response. These mechanisms go from expression of virally encoded MHC class I homologs and selective modulation of MHC class I protein expression by viral proteins to virus-mediated inhibition of activating receptor function and production of virally encoded

cytokine-binding proteins or cytokine-receptor antagonists and, finally, direct viral effects on NK cells.

A common feature of many viral infections is the virus-induced modulation of class I expression. Viruses down-modulate class I molecules that are efficient at presenting viral peptides to CD8T cells, such as HLA-A and HLA-B, to evade CTL-mediated destruction. In contrast, either HLA-C and HLA-E (described in section 1.1.4), the dominant ligands for NK cell-inhibitory receptors, are spared from virus-induced clearance from the cell surface, or their expression is specifically enhanced.

In HCMV infection at least four proteins, US2, US3, US6 and US11 act to deviate the class I molecules from their normal progression from the endoplasmic reticulum to the cell surface (described in section 1.3.1.2, 1.3.1.3). But two dominant inhibitory receptor ligands, HLA-C and HLA-E, are resistant to either US2- or US 11-mediated degradation, suggesting that virus-infected cells evade NK cell activity by sparing the class I molecules least effective at presenting viral peptides to CTL but most effective at inhibiting NK cells (Schust *et al.* 1998; Lopez-Botet *et al.* 2001). HCMV actively enhances the expression of HLA-E, the ligand for the inhibitory CD94-NKG2A receptor complex (Tomasec *et al.* 2000; Ulbrecht *et al.* 2000). Cell surface expression of HLA-E requires binding of a nanomer peptide derived from the signal sequence of most HLA molecules (Braud *et al.* 1997). The HCMV UL40 protein possesses a nanomer peptide homologous to HLA signal sequences and thus can enhance cell surface expression of HLA-E (Tomasec *et al.* 2000; Ulbrecht *et al.* 2000).

MCMV possesses three genes, *m04*, *m06* and *m152*, that selectively modulate class I expression (described in section 1.3.1.3). *m152* encodes for a 40kDa protein that blocks transport of class I molecules from the ER to the Golgi (Ziegler *et al.* 1997; Ziegler *et al.* 2000).

m04 encodes a 34kDa type I transmembrane glycoprotein which is expressed at the cell surface in association with class I molecules. *m04* it is believed to serve to oppose the action of *m152* by rescuing some class I molecules from ER retention, thus protecting infected cells from NK cells which might otherwise be activated by the loss of surface class I (Kleijnen *et al.* 1997; Kavanagh *et al.* 2001).

The *nef* gene from HIV-1, HIV-2 and the SIV, encodes a 27kDa protein that is expressed early after infection and selectively down-modulates the expression of HLA-A and HLA-B, but not HLA-C or HLA-E (Le Gall *et al.* 1998; Cohen *et al.* 1999). Thus, HIV-infected target cells remain resistant to lysis by NK cells, and their resistance depends on the failure of Nef to down-modulate HLA-C and HLA-E from the cell surface. Nef acts by inducing the clathrin adaptor protein complex to recognize a tyrosine-based sorting motif in the cytoplasmic tail of HLA-A and HLA-B (Le Gall *et al.* 1998). The resistance of HLA-C to Nef-induced down-regulation is due to locus-specific tyrosine-to-cysteine and aspartic-acid-to-asparagine substitutions in the cytoplasmic tail (Le Gall *et al.* 1998; Cohen *et al.* 1999).

NK cells, in addition to receptors with inhibitory capabilities, have receptors whose ligation can induce cytotoxicity, proliferation and cytokine production (Biassoni *et al.* 2001) (described in section 1.1.4).

An effective viral evasion strategy would be to interfere with NK cell-activating receptors. Also adhesion of the NK cell to the target cell is crucial for killing. Down-regulation of adhesion molecules on target cells has also been linked with reduced NK cell susceptibility.

In the case of human cytomegalovirus (HCMV) and Kaposi's sarcoma-associated herpesvirus (KSHV), down-regulation of the lymphocyte function-associated antigen-3 (LFA-3) and intercellular adhesion molecule-1 (ICAM-1) molecules, respectively, reduce the susceptibility of infected cells to NK cell killing (Fletcher *et al.* 1998; Ishido *et al.* 2000).

Viruses may subvert NK cell responses through virus-encoded proteins that counteract or modulate the interactions between cytokine or chemokine molecules and their cognate receptors. Numerous poxviruses and herpesviruses encode homologues of known cytokines and chemokines with agonistic or antagonistic function, or secrete proteins or receptors that bind with high affinity to cytokines and chemokines (Lalani *et al.* 2000).

Interference with anti-viral NK cell function could involve inhibition or antagonism of cytokines such as IL-2, IL-18, TNF- α , which participate in inducing NK cell IFN- γ production and cytotoxicity (Biron *et al.* 1999).

The binding and sequestration of IL-18 from its cognate receptor has been suggested to be involved in human papillomavirus-related pathogenesis. The oncoproteins HPV-16 E6 and E7 inhibit IL-18-induced IFN- γ production in phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cell (PBMC) and in an IL-12-stimulated immortalized NK cell line by specific and competitive binding to IL-18 or IL-18 α chain receptor (IL-18R α) (Lee *et al.* 2001; Cho *et al.* 2001). Down-modulation of IL-

18-induced responses by HPV oncoproteins may contribute to viral pathogenesis or carcinogenesis.

As discussed above, the selective pressure exerted by the immune system has led to the evolution of a diverse array of strategies by different viruses. A great deal of structural information is available on host targets, but for the viral proteins this analysis is only now beginning.

1.4 PROJECT AIMS

The papillomavirus E5 protein is localized in the endoplasmic reticulum (ER) and Golgi apparatus (GA) of the host cells. PalF-E5 expressing cells are enlarged and vacuolated and have a distorted Golgi apparatus (Ashrafi *et al.* 2000; Ashrafi *et al.* 2002). At least some of these characteristic are due to alkalization of the Golgi lumen (Marchetti *et al.* 2002), an effect mediated by the binding of BPV-1 and BPV-4 E5 proteins to the 16K ductin/subunit c of the V0 sector of the vacuolar H⁺-ATPase (Goldstein *et al.* 1991; Faccini *et al.* 1996). Also, this binding has been deemed responsible for the down-regulation of gap-junction communication (Faccini *et al.* 1996; Ashrafi *et al.* 2000). We asked whether all these effects would affect protein traffic in E5-expressing cells. A very important protein complex the MHC I, which is essential for presentation of foreign peptides to the cytotoxic T lymphocytes, is transported from the ER through the Golgi apparatus before reaching the plasma membrane. Many viruses have evolved strategies for evading CTL-mediate immunity by interfering with the transport and cell surface display of MHC class I (Tortorella *et al.* 2000). In E5-expressing cells the MHC class I expression is down-regulated (Ashrafi *et al.* 2002). This thesis tried to further define the relationship between PV E5 proteins, mainly BPV-4 E5 and MHC class I complex.

To this end the first aim was :

- To define whether E5 affects the transport of MHC class I molecules to the cell surface evaluating surface and total MHC I expression in E5-expressing cells

In PalF cells, BPV-4 E5 activates the transcriptional promoter of the cyclin A gene, increases the cyclin A-cdk2 activity and interfere with protein degradation

(O'Brien *et al.* 1998; O'Brien *et al.* 1999; O'Brien *et al.* 2001). In E5-expressing cells there is less MHC I HC mRNA and protein (Ashrafi *et al.* 2002).

Therefore the second aim was :

- To analyse if the MHC I heavy chain (HC) is degraded in E5-expressing cells
- To measure if inhibition of transcription of MHC I HC and its degradation can be alleviated by IFN treatment
- To evaluate if E5 interacts physically with HC and which domain is involved
- To determine if this physical interaction with HC is common to other E5 protein such as BPV-1 E5 or HPV-16 E5

Virus infected cells lacking classical MHC I on their surface would be killed by NK cells, unless non-classical MHC I molecules are present. Many viruses have evolved strategies to selectively down-regulate classical but not non-classical MHC I.

The third aim was:

- To evaluate if E5 interact with non-classical MHC I

The data presented in this thesis add a new step in the understanding of general downregulation of MHC class I by E5 and point to a common pathway of immunevasion by E5 proteins from different PV types.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Antibodies

SUPPLIER	ANTIBODY
Merck Biosciences Ltd Nottingham, UK	Anti-Actin (Ab-1). Mouse monoclonal IgM antibody (clone JLA20).
Amersham Pharmacia Biotech, Amersham, Bucks, UK	Anti-mouse IgG horseradish peroxidase linked whole antibody (raised in sheep)
Sigma Chemical Co., Ltd., Poole, Dorset, UK	Anti-mouse IgG (Fab specific)-FITC conjugated (raised in goat) specific for flow cytometry
Sigma Chemical Co., Ltd., Poole, Dorset, UK	Anti-mouse IgG (whole molecule) -TRITC conjugated (raised in rabbit) specific for immunofluorescence
Sigma Chemical Co., Ltd., Poole, Dorset, UK	Anti-mouse IgG (whole molecule)-FITC conjugated (raised in sheep) specific for immunofluorescence
Cambridge Biosciences, Cambridge, UK	Anti-mouse IgG-FITC conjugated (raised in goat) AlexaFluor 488 specific for immunofluorescence.
Sigma Chemical Co., Ltd., Poole, Dorset, UK	Anti-Rat IgG (whole molecule) peroxidase conjugate (raised in rabbit)
AbCam Ltd, Cambridge, UK	4A3, a mouse monoclonal antibody raised against the 97kDa fragment of GM130 a member of the Golgin protein family localised in the Golgi.
Cambridge Biosciences, Cambridge, UK	BODIPY [®] TR Ceramide is a red-fluorescent dye that is used to stain the Golgi apparatus.

VMRD, Inc. Pullman, WA 99163, U.S.A.	H58A, a mouse monoclonal antibody raised against the equine MHC class I
Covance 800 University Avenue Berkeley, California 94710-2021	HA.11, a mouse monoclonal antibody raised against the twelve amino acids peptide C(YPYDVPDYA)SL. It recognizes the influenza hemagglutinin epitope in bracket.
A kind gift from Dr. Stephen Man, Cardiff University, Cardiff, U.K.	HC10, a mouse monoclonal antibody specific for HLA-A,-B,-C class I heavy chain.
Vh Bio Ltd. U.K.	H-2Ld, a mouse monoclonal antibody specific for the mouse MHC class I.
A kind gift of Dr Jan Naessens International Livestock Research Institute Nairobi, Kenya	IL-A165, a mouse monoclonal antibody that recognizes the bovine transferrin receptor.
A kind gift of Dr Liz Glass (IAH, Roslin, UK)	IL-A19, a mouse monoclonal antibody raised against the bovine MHC class I molecules (monomorphic determinant), recognizes β 2-microglobulin-associated MHC I heavy chain.
A kind gift of Dr Shirley Ellis (IAH, Compton, UK)	IL-A88, a mouse monoclonal antibody raised against the bovine MHC class I molecules (monomorphic determinant) recognizes the MHC I free heavy chain.
Animal Health Trust (Suffolk, UK)	MAC 291, a rat monoclonal antibody raised against equine MHC class I
A gift from Dr. M. Hibma, ICRF Tumour Virus Group, University of Cambridge	TVG261, a mouse monoclonal antibody directed against amino acids 2–17 in the amino terminus of HPV-16 E2.

2.1.2 Bacterial Hosts

SUPPLIER	BACTERIAL HOSTS
Invitrogen Life Technologies, Ltd., Paisley, UK	<i>E. coli</i> DH5α competent cells
Stratagene California Building Hogehil Weg 15 Amsterdam, Netherlands	XL1-Blue competent cells

2.1.3 Buffers

TE	10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0
10× TBE buffer	900mM Tris base, 900mM boric acid, 25mM EDTA, pH8.0
Phosphate buffered saline (PBS)	137mM NaCl, 44mM KCl, 1.4 mM KH ₂ PO ₄ , 8.5 mM Na ₂ HPO ₄
Tris buffered saline (TBS)	40mM Tris HCl pH 7.6, 1.4M NaCl
Fixing Buffer	50% (w/v) Methanol, 40%(w/v) Glacial Acetic Acid
10 × loading buffer	65% (w/v) Sucrose, 10mM Tris HCl pH8, 10mM EDTA pH8, 0.3% (w/v) Bromophenol blue
SDS-PAGE Lysis buffer	100mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 2% (w/v) glycerol
2x SDS gel loading buffer	4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol and 100mM Tris, pH6.8
RIPA buffer	50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1% Nonidet® P40, 0,5% sodium deoxycholat, 0.1% SDS, protease inhibitors

2.1.4 Cells

CELL TYPES	DESCRIPTION	GROWTH MEDIUM
PalF	Primary embryonic bovine fibroblasts and transformed one described in sections 2.2.2.1.2 and 2.2.2.1.3	Dulbecco's Modified Eagles Medium, 10% Foetal Calf Serum 4mM L-glutamine 100 IU penicillin 100µg streptomycin
NIH 3T3	Mouse fibroblasts	Dulbecco's Modified Eagles Medium, 10% Foetal Calf Serum 4mM L-glutamine 100 IU penicillin 100µg streptomycin
P815	Mouse mastocytoma cell line	RPMI 1640 Medium 10% Foetal Calf Serum 100 IU penicillin 100µg streptomycin

2.1.4.1 Cell Culture Materials

SUPPLIER	MATERIAL
Invitrogen Life Technologies, Ltd., Paisley, UK	Foetal Calf Serum
Invitrogen Life Technologies, Ltd., Paisley, UK	10% Dulbecco s Modified Eagles Medium 200 mM glutamine Geneticin. G418 sulphate 100 mM sodium pyruvate Trypsin (1x)

2.1.5 Chemicals and Enzymes

Supplier- Amersham International plc, Amersham, Bucks, UK

ECL Western detection agent

ECL Plus Western detection agent

Hyperfilm ECL

Redivue™ L-[³⁵S] methionine

Amplify™ Fluorographic reagent

Supplier- BDH Chemicals Ltd., Poole, Dorset, UK

Calcium chloride

D-glucose

Glycerol

Supplier- Beta Lab., East Mosley, Surrey, UK

Yeast Extract

Supplier- Calbiochem, San Diego, U.S.A.

ALLN (Calpain Inhibitor I)

Bafilomycin A1

Concanamycin A (Folimycin)

LY 294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one]

Leupeptin hemisulfate

MG-132 (Carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal)

NH₄Cl

Supplier- Citifluor Ltd.UK

AF1 Citifluor

Supplier- Difco laboratories, Detroit, Michigan, USA.

Bacto-Agar

Bactotryptone

Supplier- Invitrogen Life Technologies, Ltd., Paisley, UK

All DNA restriction enzymes and appropriate buffer concentrates were obtained from Invitrogen Life Technologies unless otherwise stated. The following reagents were also obtained from Invitrogen:

Agarose (ultrapure electrophoresis grade)

LIPOFECTAMINE™ Reagent

LipofectAMINE PLUS™ Reagent

Nitrocellulose membrane filter paper sandwich 0.45µm pore size

NuPAGE® Novex® 4-12% Bis-Tris Gel

Novex® 4-12% Tris-Glycine Gel

10x Novex® Tris-Glycine Running Buffer

20x NuPAGE® MES SDS Running Buffer

20x NuPAGE® MOPS SDS Running Buffer

20x NuPAGE® Transfer Buffer

4x NuPAGE® LDS Sample Buffer

2x Novex® Tris-Glycine SDS Sample Buffer

NuPAGE® Sample Reducing Agent

NuPAGE® Antioxidant buffer

T4 DNA ligase

Supplier- James Burrough Ltd., Witham, Essex, UK

Ethanol

Supplier- New England Biolabs

Alkaline phosphatase Calf Intestinal (CIP)

Bovine Serum Albumin

Supplier- Promega, Southampton, UK

TNT® Quick Coupled Transcription/Translation System

Canine Pancreatic Microsomal Membranes

Supplier- Sigma Chemical Co., Ltd., Poole, Dorset, UK

β-mercaptoethanol

Bicinchoninic Acid solution
Bovine Serum Albumin
Bromophenol Blue
Coomassie Brilliant Blue R-250
Copper(II) sulphate (pentahydrate 4% (w/v) solution)
DEPC
Dithiothreitol (DTT)
Di-potassium hydrogen orthophosphate anhydrous
Ethylene diamine tetra acetate (EDTA) disodium salt
Dimethyl sulfoxide (DMSO)
Ethidium Bromide
Kanamycin
Kodak X-OMAT AR film
Leupeptin
Magnesium chloride
Magnesium sulphate
Monensin
Nonidet P-40 (NP40)
Phenol:Chloroform:Isoamyl Alcohol (25:24:1 (v/v))
Ponceau S solution
Potassium chloride
Potassium dihydrogen orthophosphate
Protein G-Sepharose suspension
Saponin
Sodium acetate
Sodium carbonate
Sodium dodecyl sulphate (SDS)
Sodium hydroxide
Tween-20 (Polyoxyethylene sorbitan nonolaurate)

Supplier- Roche Diagnostics GmbH, Germany

Protease Inhibitor Cocktail tablets

Supplier Vector Laboratories Inc, UK

Vectashield

Supplier- VH Bio, Gateshead,UK

Performa® DTR Gel Filtration Cartridges

Supplier- VWR International, Leicestershire,UK

Acetic acid

Butan-1-ol

Chloroform

Hydrochloride acid

Methanol

Propan-2-ol

Sodium chloride

Tris Base

2.1.6 Equipment and Plasticware

SUPPLIER	EQUIPMENT
Becton Dickinson Labware, Plymouth, UK	Falcon 2059 polypropylene tubes Falcon 2098 polypropylene tubes Sterile Plastipak syringes 18 gauge sterile syringe needles 60, 100 mm tissue culture dishes
Greiner	Sterile bijoux tubes Sterile universal tubes Cell scrapers Filter tips 60 and 100mm bacteriological petri dishes Sterile plastic universal containers
Corning BV, High Wycombe, Bucks, UK	25ml, 10ml 5ml pipettes 24 well tissue culture plates 96 well tissue culture plates Cryogenic vials

VMR (MERCK/BDH)	Sterile syringes Needles Saran wrap Foil
Scientific Laboratory Supplies	T25, 80, and 175 cm ² tissue culture flasks
Sartorius	Sterile 0.2µm filter Sterile 0.45µm filter
Labco	Cin Bins
Whatman International Ltd., Maidstone, Kent, UK	Whatman 3MM filter paper

2.1.7 Kits

SUPPLIER	KIT
Perkin Elmer Cetus, Norwalk, USA.	GeneAmp thinwalled reaction tubes
Applied Biosystems, Cheshire, UK	BigDye™ Terminator v3.1 Cycle Sequencing BigDye™ 5x Sequencing Buffer HI-DI formamide Taqman EZ RT-PCR kit
Promega Ltd., Chilworth Research Centre, Southampton, UK	TNT® Quick Coupled transcription/translation system Canine Pancreatic Microsomal Membranes
Qiagen Ltd., Dorking, Surrey, UK	QIA prep Spin plasmid miniprep kit QIAquick gel extraction kit QIAquick PCR purification kit QIAGEN plasmid maxi kit RNAeasy mini kit

2.1.8 Molecular Weight Markers

SUPPLIER	MARKER
Invitrogen Life Technologies, Ltd., Paisley, UK	See Blue ® Plus 2 Pre-Stained Protein Standard
Invitrogen Life Technologies, Ltd., Paisley, UK	φX174 RF DNA/Hae III Fragments 100bp DNA ladder 1Kb DNA Ladder

2.1.9 Other Materials

SUPPLIER	MATERIALS
Veterinary Pathology Central Services	LB-Medium (Luria-Bertani Medium) SOC Sterile distilled water Sterile glycerol Sterile phosphate-buffered saline (PBS)
Merck Ltd., Poole, UK	Silicone grease
Johnson and Johnson Medical Limited, Berks, UK	PRESEPT* effervescent disinfectant tablets.
Premier Beverages., Adbaston, Stafford, UK	Marvel (Dried Skimmed milk)

2.1.10 Plasmids

The constructs described in this section were all generated by myself unless otherwise stated. All constructs were verified by sequencing as described in section 2.2.3.1. For a schematic representation refer to section 2.1.11. The constructs are listed in alphabetical order.

pBluescript II SK (+) is a 3.0Kb cloning vector obtained commercially from Stratagene.

pBluescript-HLA-A2 (pBS-A2) contains the HLA-A2 gene cut out from pAL356-A2 (kind gift of Dr Steve Mann) and inserted in the EcoRV/SpeI sites of pBluescript II SK (+) under the control of the T7 promoter.

pBoLa-Luc was derived from pBoLa-19 (kind gift from Dr G. Russell), a plasmid containing the promoter/enhancer, exon 1 and the 3'UTR of a cattle MHC class I allele (Sawhney *et al.* 1995; Sawhney *et al.* 1996). The promoter sequences were amplified by PCR using the forward primer

5'-GTTGAAGGCTCTCGAGGGCATCGGTCGAC-3' and the reverse primer

5'-TGCAAAGCTTCCTCTGGGTCTGGGAAGAAGC-3'. The resulting amplicon of 1100 bp was digested with XhoI/HindIII and inserted between the XhoI/HindIII sites of pGL3 (Promega) ahead of the luciferase gene sequence. (This construct was made by Dr Robina Ullah, a former worker of this laboratory).

pCI-neo16E5 contains the full length HPV-16 E5 open reading frame inserted in the NheI/XhoI sites of the commercial cloning vector pCI-neo (Promega). This plasmid was a kind gift of Prof. Angel Alonso.

pCMV-E2 expresses the full length E2 protein of HPV-16 (nt 2725 to 3852) cloned into the XbaI/SmaI sites of the cytomegalovirus immediate-early promoter/enhancer-based expression vector pCMV₄ and was a kind gift from Dr Lawrence Bank, ICGEB, Trieste, Italy).

pcDNA3 is a 5.4 Kb cloning vector obtained commercially from Invitrogen. This plasmid can be used as a standard cloning vector, as a template for *in vitro* transcription under the control of T7 or Sp6 promoters.

pcDNA3-N*01301 contain the *Bos taurus* cDNA for a MHC class I allele N*01301 (Formerly known as HD6 (Ellis *et al.* 1996) GenBank accession number:X80934) inserted in the HindIII/EcoRI sites of the commercial cloning vector pcDNA3 (kind gift of Dr Shirley Ellis).

pcDNA3-N*01301Stop₃₃₉ is a construct that consists of a truncated form of N*01301 in which a premature stop codon has been introduced at residue 339.

This mutant was generated by PCR using as template the pcDNA3-N*01301 with the following primers:

Forward T7: 5'-CGAAATTAATACGACTCACTATAGGGAGACCCAAGC-3'

N*01301Stop: 5'- CTGTCATTGCTTGCAGCCTCGAGCTAGGTTGTAGGTCCGTC -3'

The reverse primer adds a point mutation resulting in a stop codon in position 339 (CAG in TAG) and an XhoI site (underlined) downstream the cloned sequence. The resulting PCR product was then HindIII/XhoI digested and inserted in the HindIII/XhoI sites of commercial vector pcDNA3.

pcDNA3-N*00201 contain the *Bos taurus* cDNA for a MHC class I allele N*00201 (Formerly known as JSP.1 (Pichowski *et al.* 1996) GenBank accession number:X92870) inserted in the XbaI/EcoRI sites of the commercial cloning vector pcDNA3 (kind gift of Dr Shirley Ellis). N*01301 and N*00201 are two different alleles of class I loci.

pcDNA3-B2 contains the equine cDNA for equine class I allele B2 (GenBank accession number: X79891) inserted in the EcoRI site of a commercial cloning vector pcDNA3 (kind gift of Dr Shirley Ellis).

pcDNA3-B4 contains the equine cDNA for equine class I allele B4 (GenBank accession number:X79892) inserted in the EcoRI site of a commercial cloning vector pcDNA3 (kind gift of Dr Shirley Ellis).

pcDNA 3.1 (-) is a 5.4kb vector derived from pcDNA3 and designed for high-level stable and transient expression in mammalian hosts also as a template for *in vitro*

transcription under the control of a T7 promoter. Obtained commercially from Invitrogen.

pcDNA 3.1 (-)/HA4E5 is a construct that consists of the BPV-4 DNA fragment nt 332 to 460 encoding the E5 protein, tagged upstream with the HA peptide, (cut out from pRFP-HAE5 177bp with BglII/HindIII) inserted into the BamHI/HindIII sites of pcDNA 3.1(-).

pcDNA 3.1(-)HA-N17Y is a construct that consist of the N-terminus HA tagged BPV-4 E5 protein containing a single amino acid substitution at residue 17 in which an asparagine has been changed to tyrosine. This mutant was cut out from pZipneo with BamHI and inserted in the BamHI site in pcDNA3.1(-).

pcDNA 3.1(-)HA-N17A is a construct that consists of the N-terminus HA tagged BPV-4 E5 protein containing a single amino acid substitution at residue 17 in which an asparagine has been changed to alanine. This mutant was cut out from pZipneo with BamHI and inserted in the BamHI site in pcDNA3.1(-).

pcDNA 3.1(-)HA-4E5T is a construct that consists of a truncated form of N-terminus HA tagged BPV-4 E5 protein in which a premature stop codon has been introduced at residue 33. This mutant form was cut out from pZipneo with BamHI and inserted in the BamHI restriction site in pcDNA3.1(-).

pcDNA3.1(-)HA-1E5 (1E5) is a construct that consists of the full length BPV-1 E5 protein, tagged upstream with the HA peptide, cut out from pZipneo-HA1E5 with BamHI and inserted into the BamHI of pcDNA 3.1(-).

pcDNA3.1(-)HA-1E5T (1E5T) is a construct that consists of a truncated form of N-terminus HA tagged BPV-1 E5 in which a premature stop codon has been introduced at residue 32.

This mutant was generated by PCR using as template the pcDNA3.1(-)HA1E5 with the following primers:

Forward BamHI: 5'-CCACCACACTGGACTAGTGGATCCACTATGTACCC-3'

RevHindIIIStop: 5'- GCACTCAAAATGATCAAGCTTCTAGTATACAAGAAAAACAGC -3'

The forward primer maintains the BamHI restriction site (underlined) upstream of the cloned sequence and the reverse primer adds a point mutation resulting in a stop codon in position 32 (TGG in TAG) and an HindIII site (underlined) downstream of the cloned sequence. The resulting PCR product was then BamHI/HindIII digested and inserted in the BamHI/HindIIIsites of pcDNA3.1(-).

pcDNA6-N*50001-V5-His contain the *Bos taurus* cDNA for an MHC class I of unknown function (Formerly known as HD59 (Ellis *et al.* 1996) or GeneX (Holmes *et al.* 2003) GenBank accession number:AY188807) cloned into the HindIII/XhoI sites of pcDNA6/V5-His ABC (Invitrogen) (kind gift of Dr Shirley Ellis).

pcDNA6-N*01301-V5-His contain the *Bos taurus* cDNA for a MHC class I allele (GenBank accession number:X80934) inserted in the HindIII/XhoI sites of the

commercial cloning vector pcDNA6/V5-His ABC (Invitrogen) (kind gift of Dr Shirley Ellis).

pDsRed-N1 is an eukaryotic expression plasmid for the Red Fluorescent Protein (RFP). RFP expression is driven by the CMV immediate early promoter and the multiple cloning site is between the RFP sequence and SV40 poly A site. It is commercially available from Clontech.

pEGFP-C1 is an eukaryotic expression plasmid for the Green Fluorescent Protein (GFP). GFP expression is driven by the CMV promoter and the multiple cloning site is between the GFP sequence and SV40 poly A site. It is commercially available from Clontech Lab. This plasmid was a kind gift from Dr M. Roberts (ICM, Glasgow).

pGEM®-11Zf(+) is a 3.2Kb vector obtained commercially from Promega. This plasmid can be used as a standard cloning vector, as a template for *in vitro* transcription and for the production of ssDNA.

pGEM/HLA-A1 contain the HLA-A1 gene, cut out from pRSV5neoHLA-A1 (kind gift of Dr Steve Mann), and inserted in the SalI/BamHI sites of pGEM –11Zf(+) under the control of the T7 promoter.

pGEM/HLA-B8 contains the HLA-B8 gene, cut out from pRSV5neoHLA-B8 (kind gift of Dr Steve Mann), and inserted in the Sall/BamHI sites of pGEM -11Zf(+) under the control of the T7 promoter.

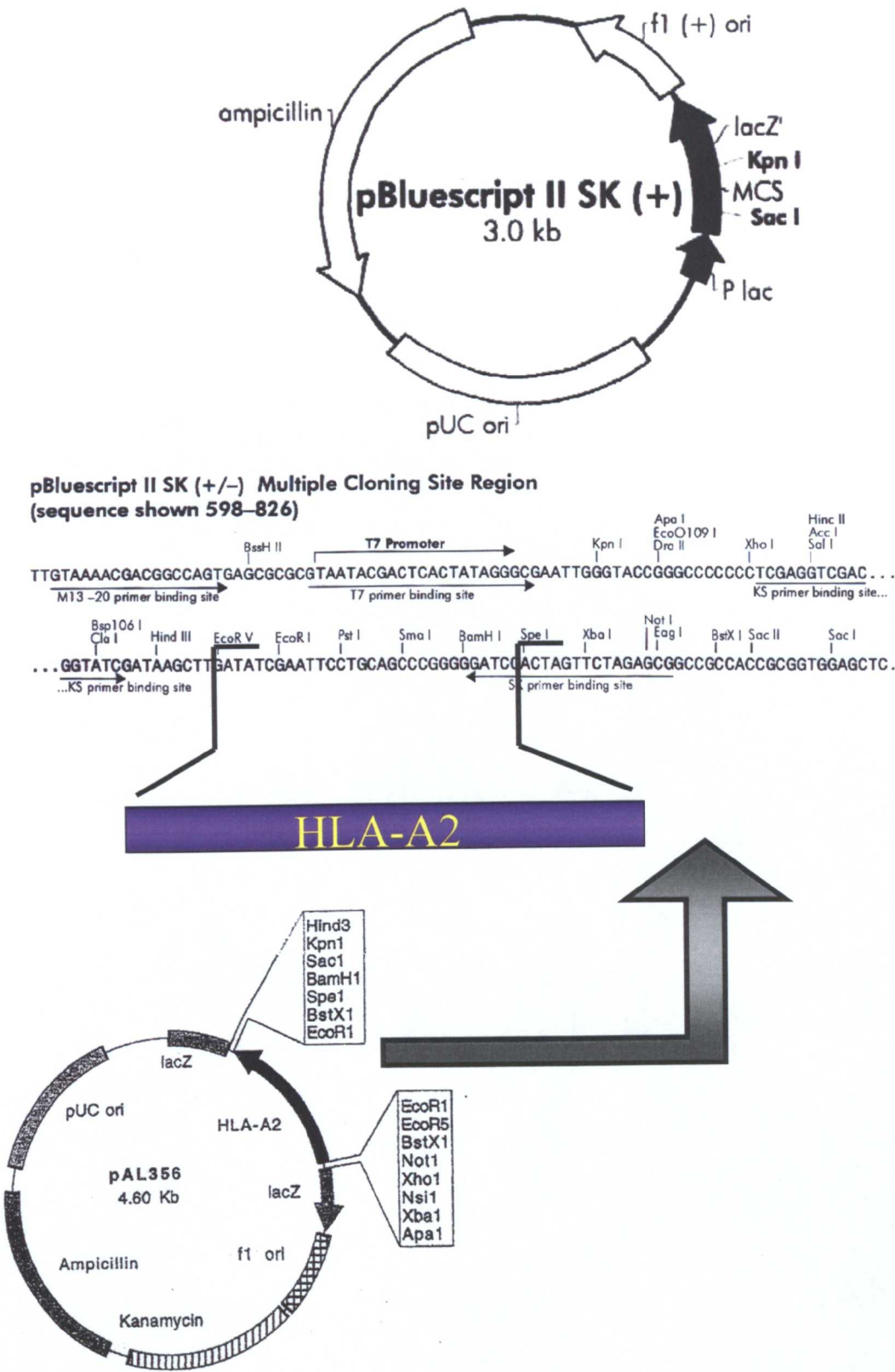
pGFP-MHC I expresses a fusion protein made up of the green fluorescent protein (GFP) from pEGFP-C1 (Clontech) and the heavy chain of human MHC I B2705 inserted in the Hind III site of pRc/RSV vector (Invitrogen) and was a kind gift from Dr Simon Powis (University of Dundee, UK). In this construct the MHC I is tagged at its C-terminus with the GFP.

pRFP-HA4E5 expresses a fusion protein made up of the red fluorescent protein (DsRed) and the HA tagged BPV-4 E5 open reading frame inserted in BglII/HindIII sites of pDsRed-N1 vector (Clontech). (This construct was made by Emma Tsirimonaki, a former worker of this laboratory).

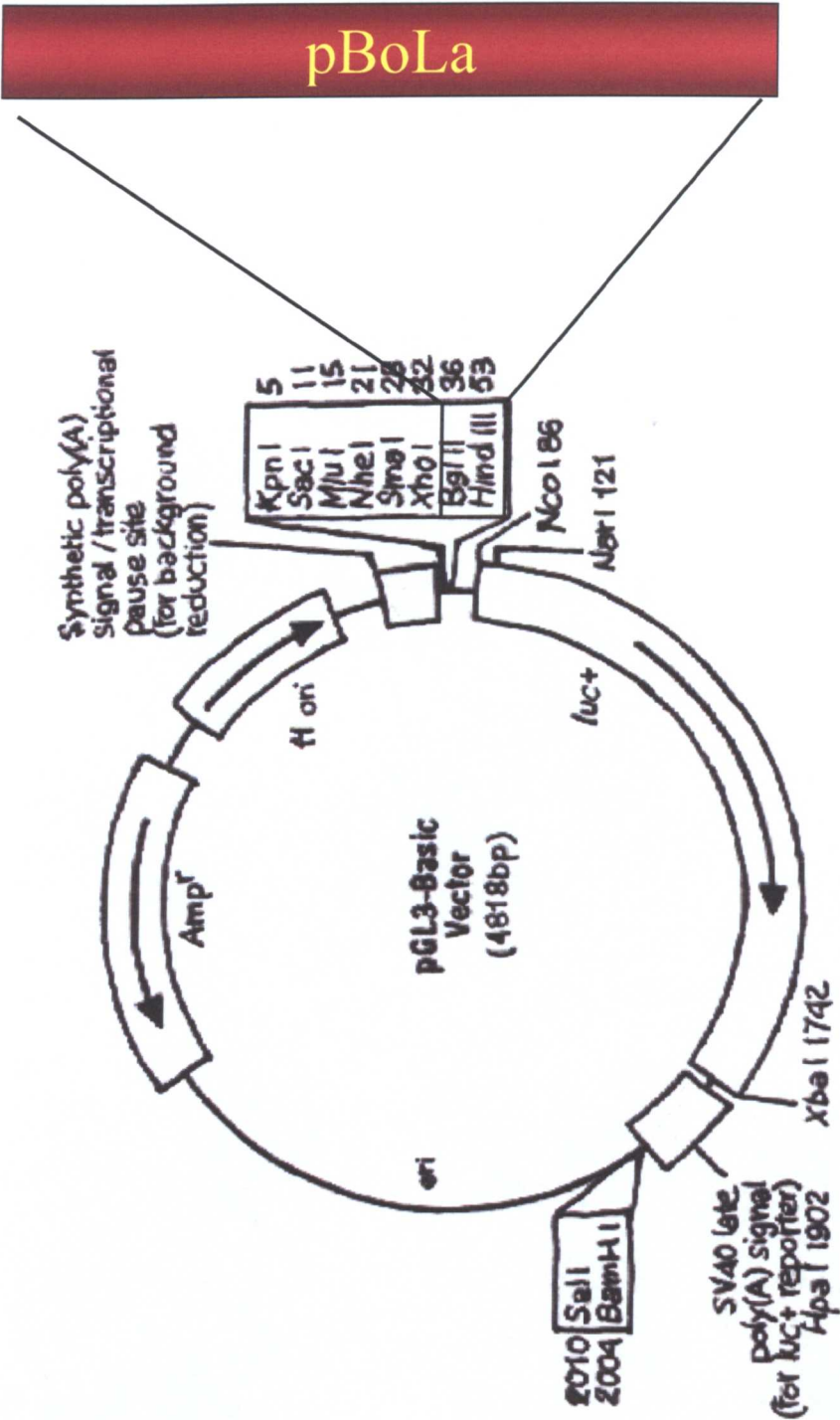
2.1.11 Schematic representation of constructs used in this thesis

For text description refer to section 2.1.10
The constructs are listed in alphabetical order

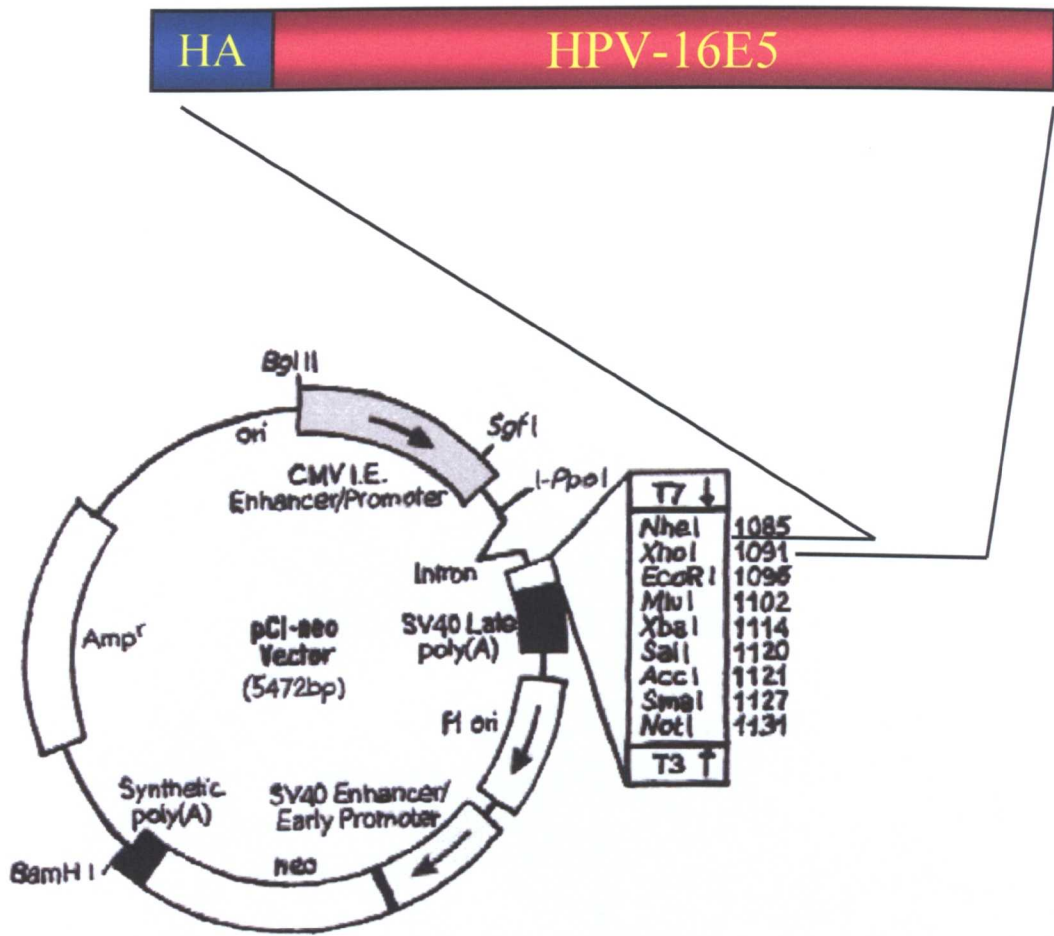
pBluescript-HLA-A2 (pBS-A2)



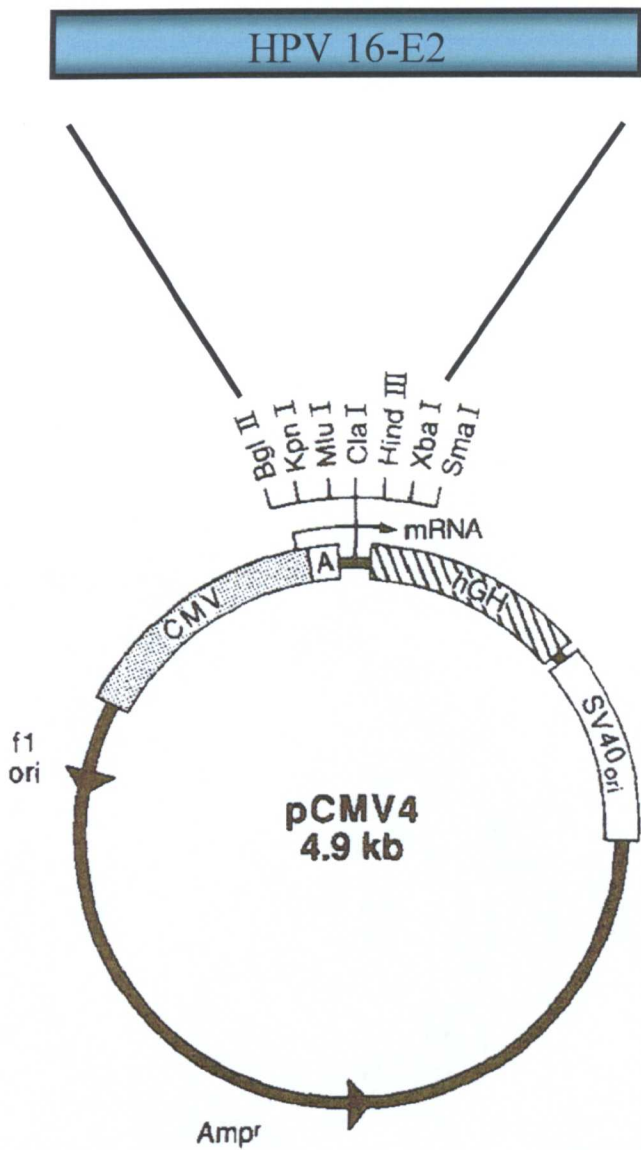
pBoLa-Luc



pCI-neo16E5

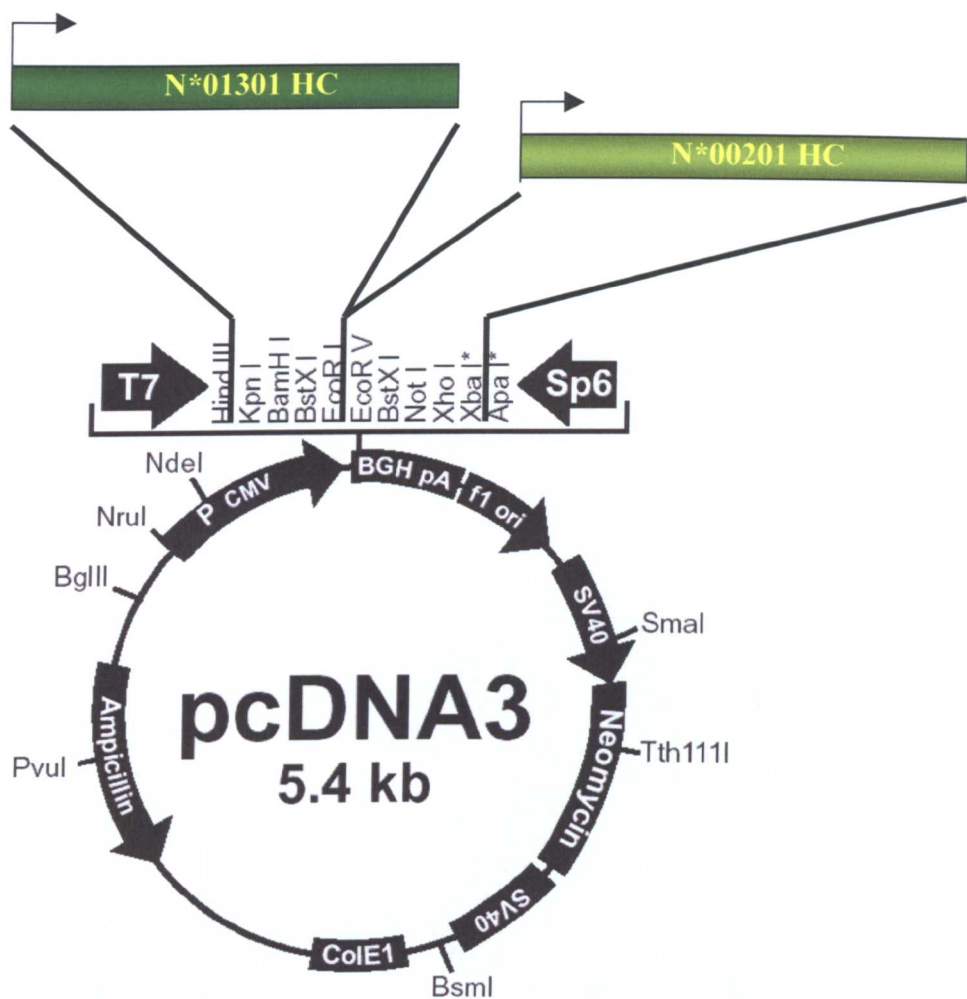


pCMV-E2

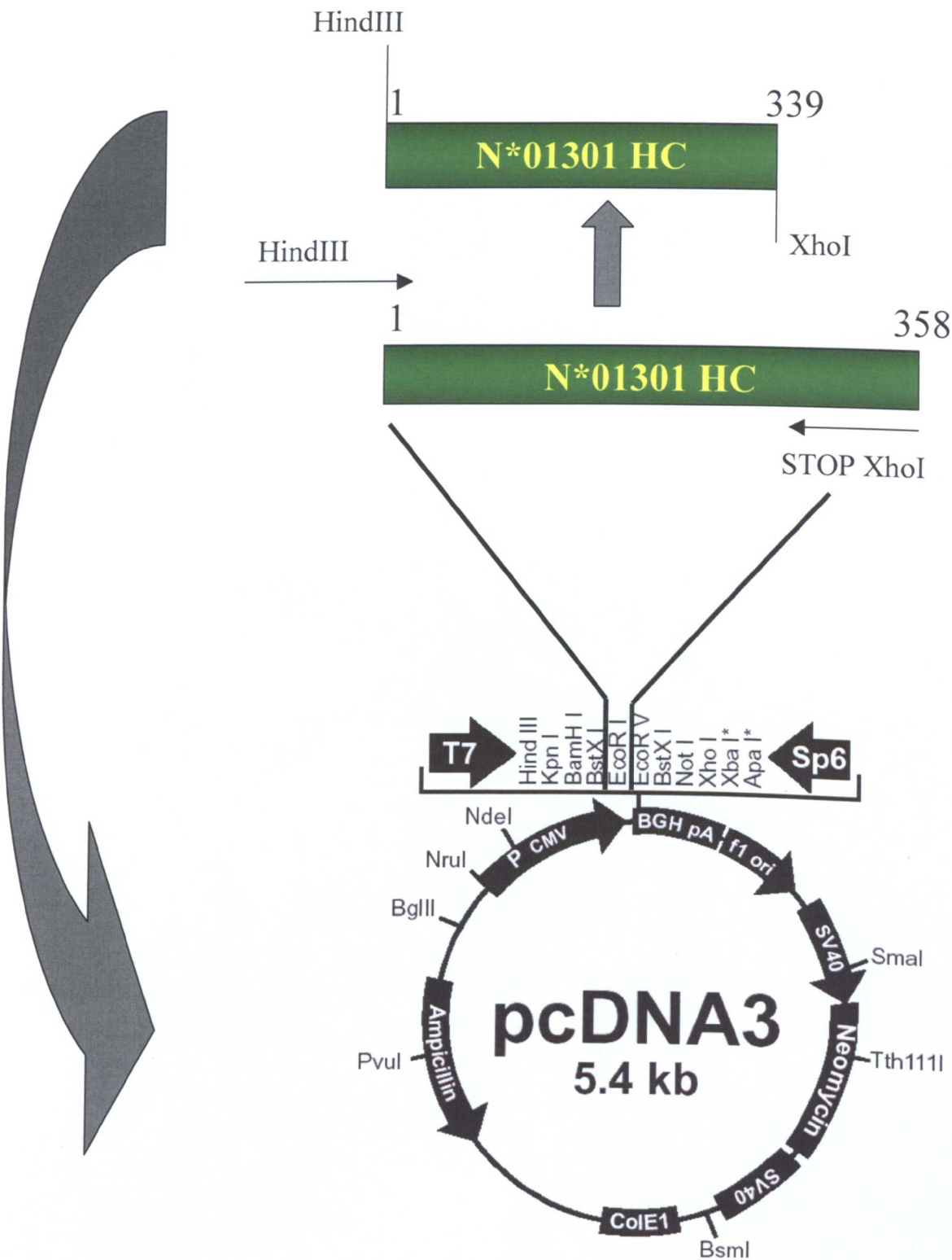


Schematic representation
adapted from data by
Anderson *et al.*, 1989
Del Vecchio *et al.*, 1992

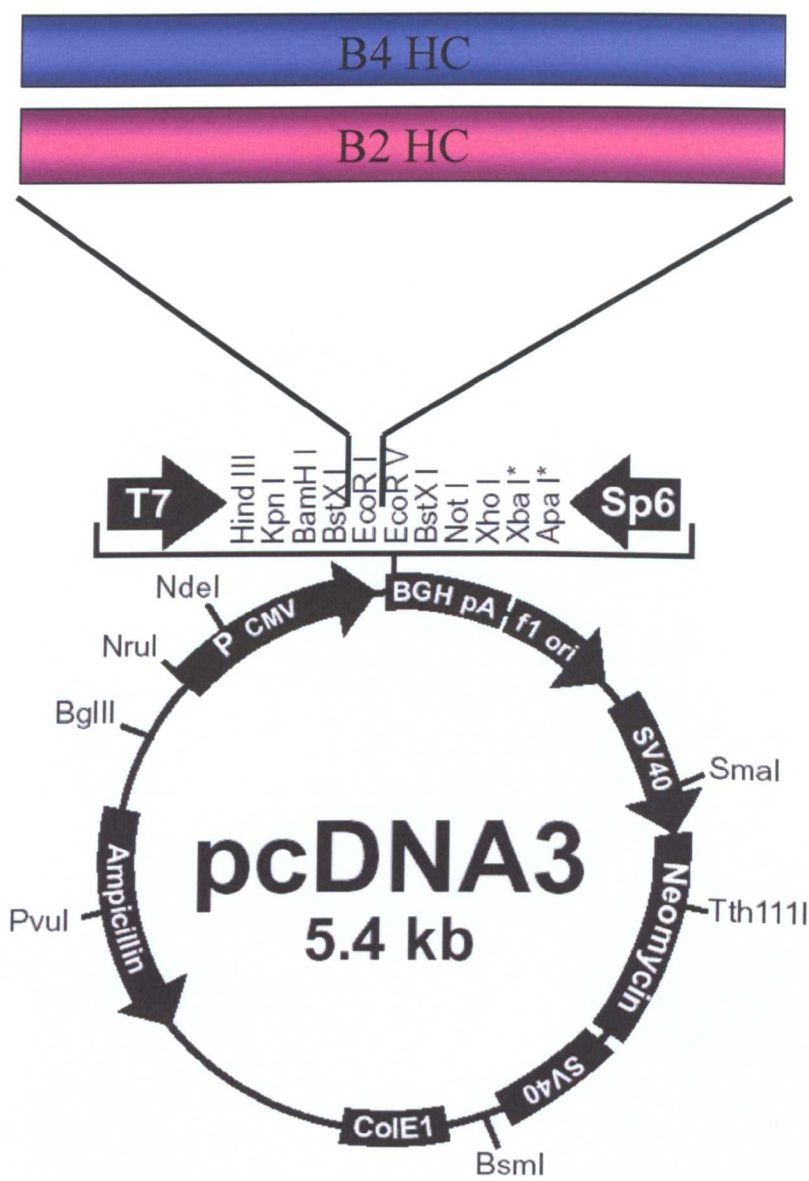
pcDNA3-N*01301
pcDNA3-N*00201



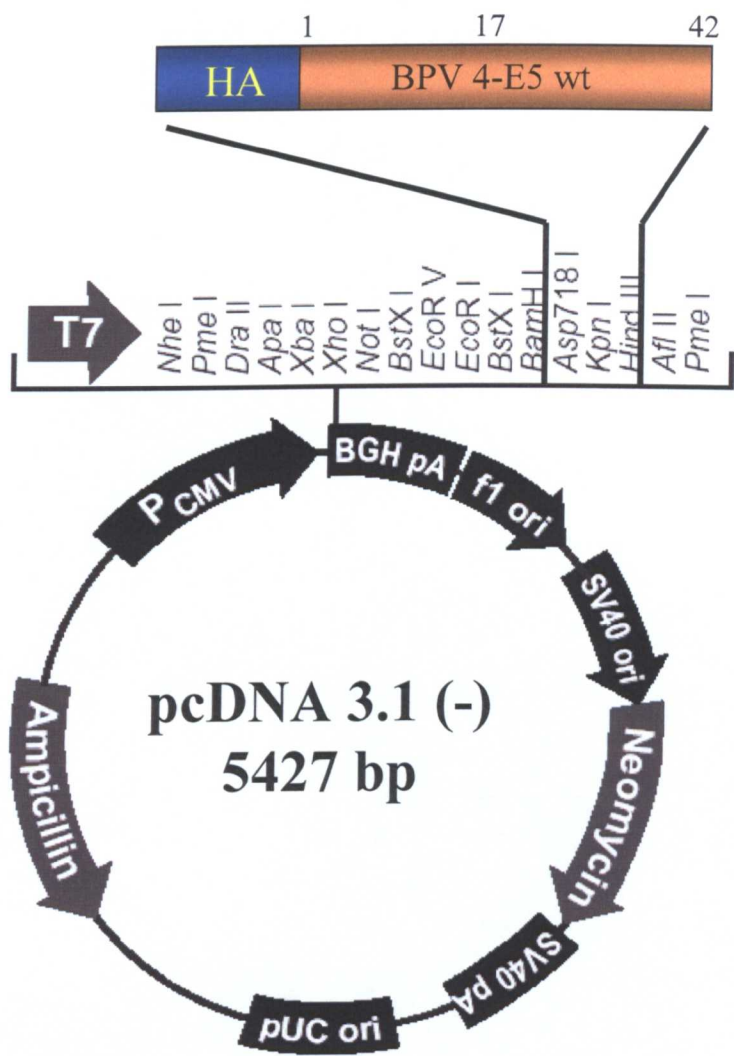
pcDNA3-N*01301Stop₃₃₉



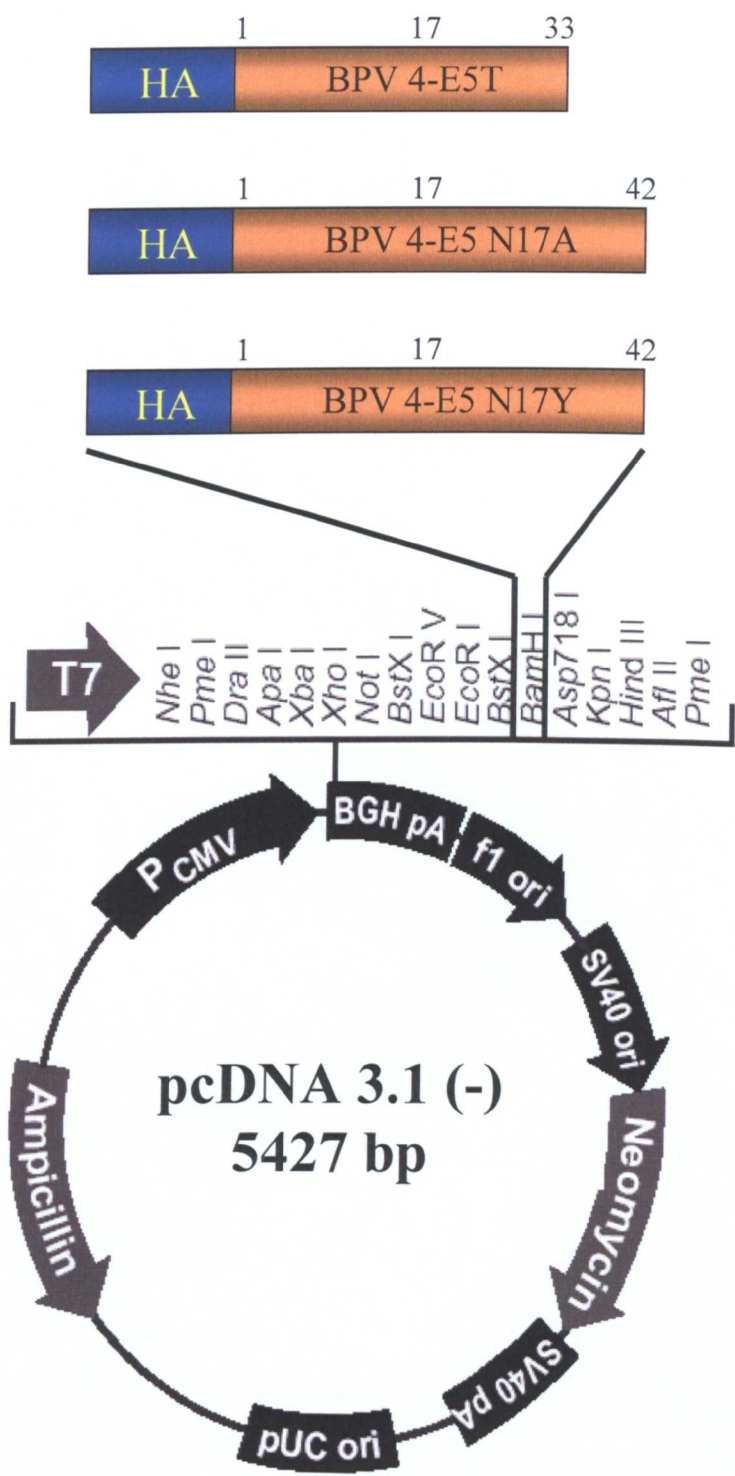
pcDNA3-B2
pcDNA3-B4



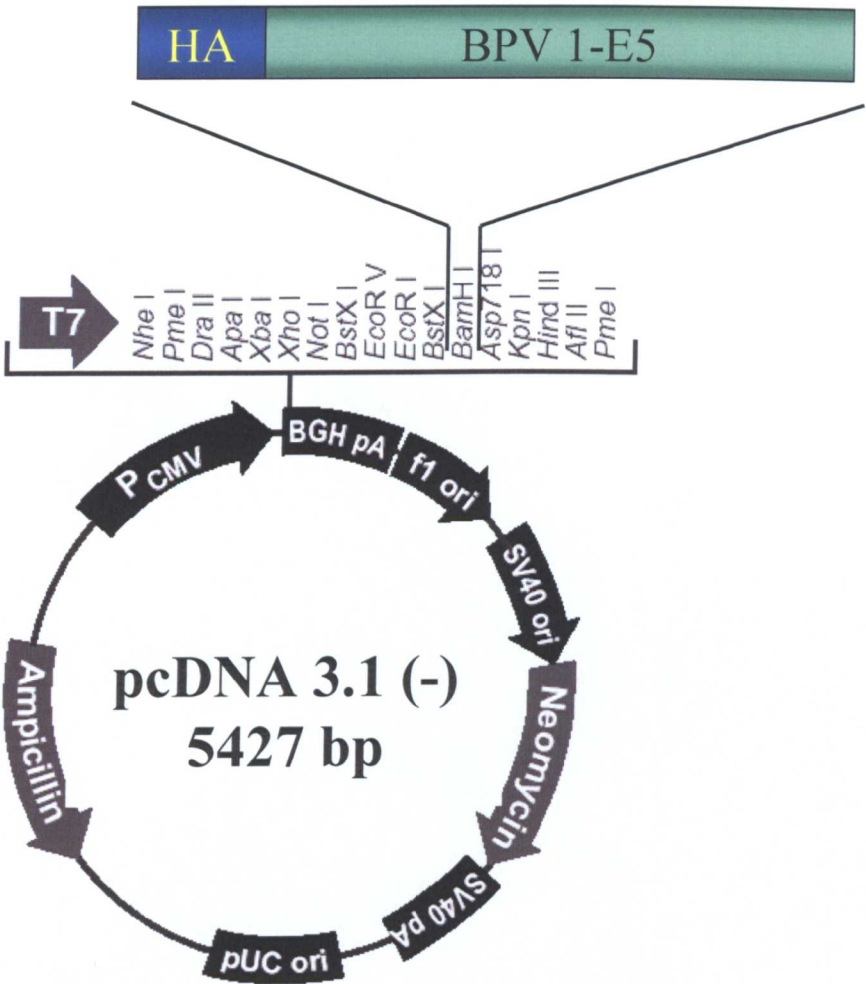
pcDNA3.1(-)HA4E5



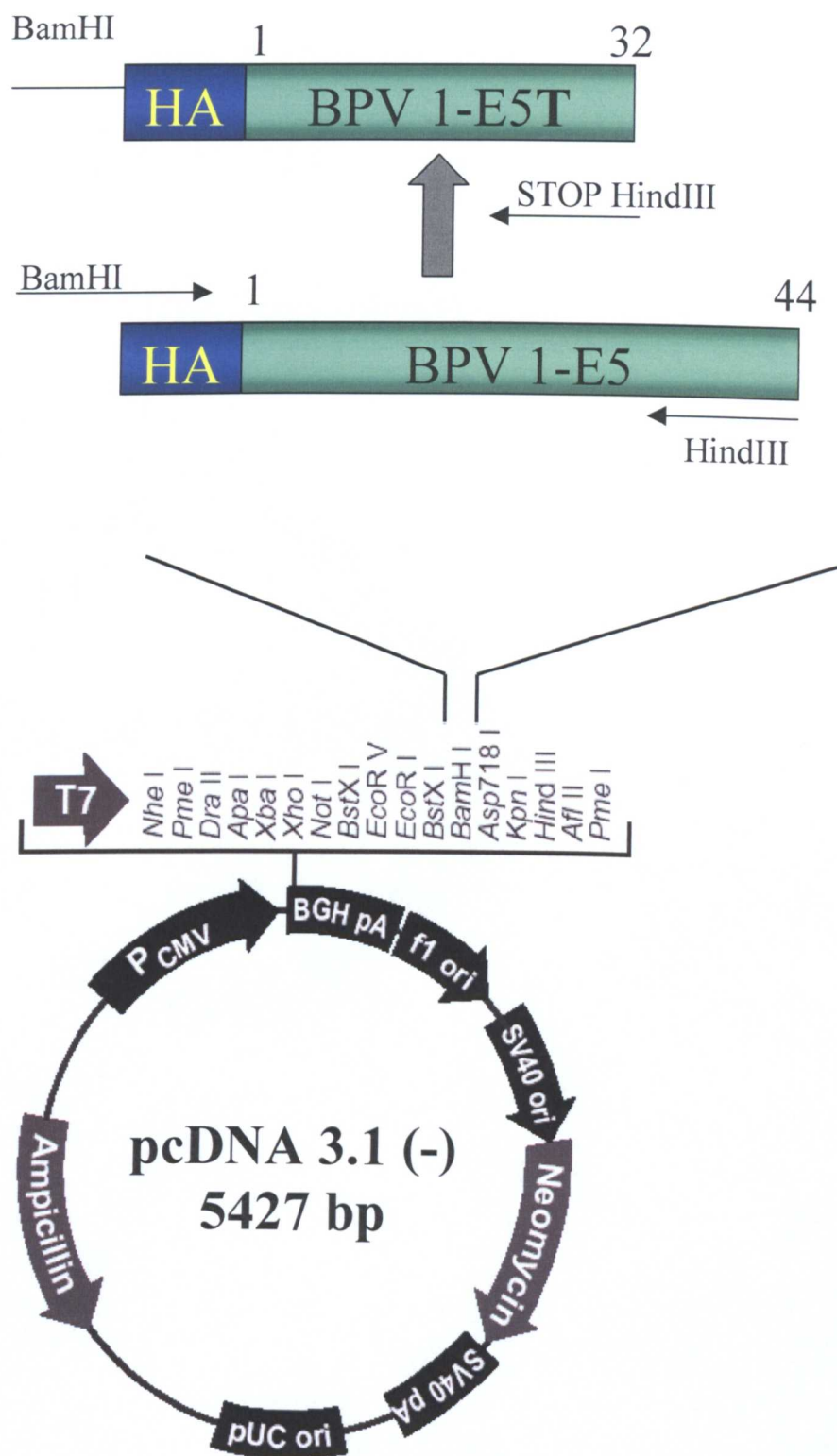
pcDNA3.1(-)HA-4E5T
pcDNA3.1(-)HA-N17A
pcDNA3.1(-)HA-N17Y



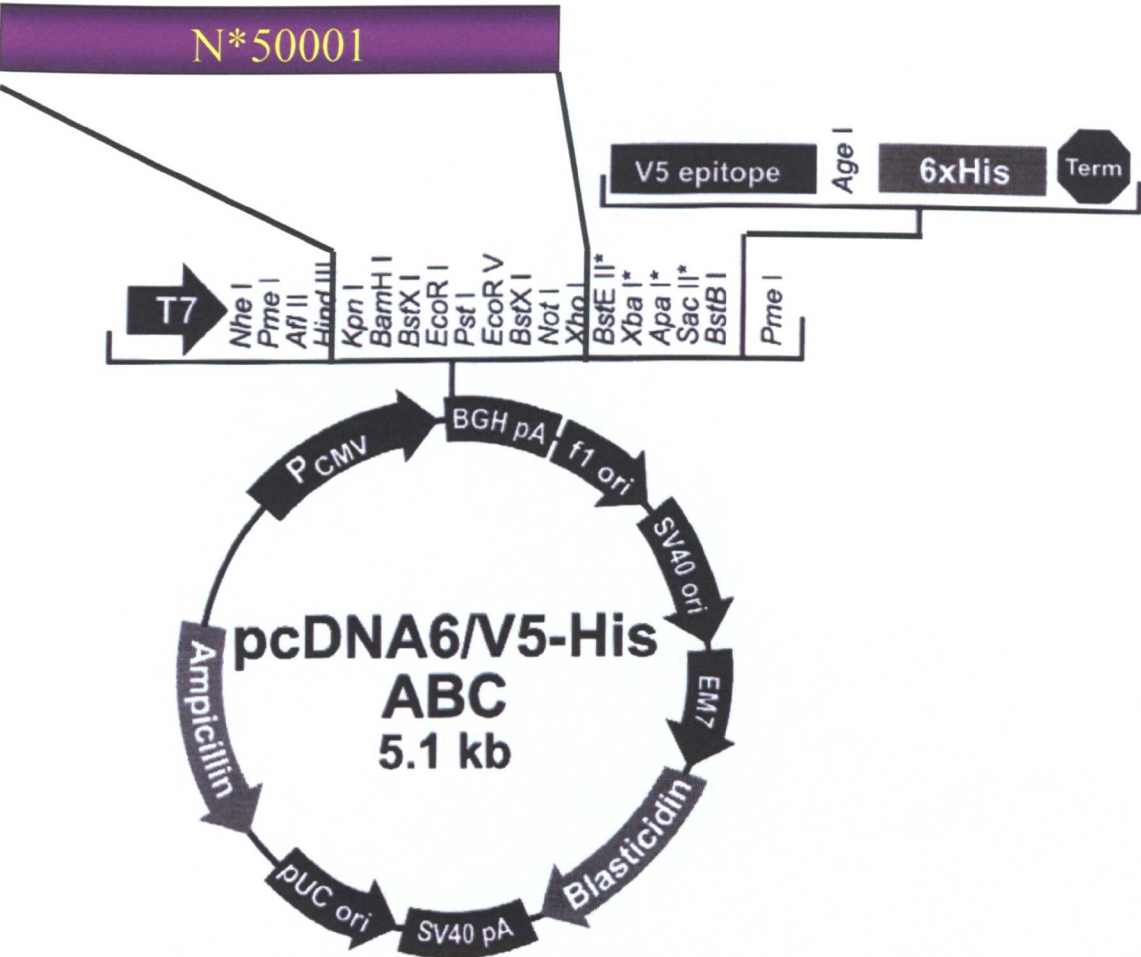
pcDNA3.1(-)HA-1E5



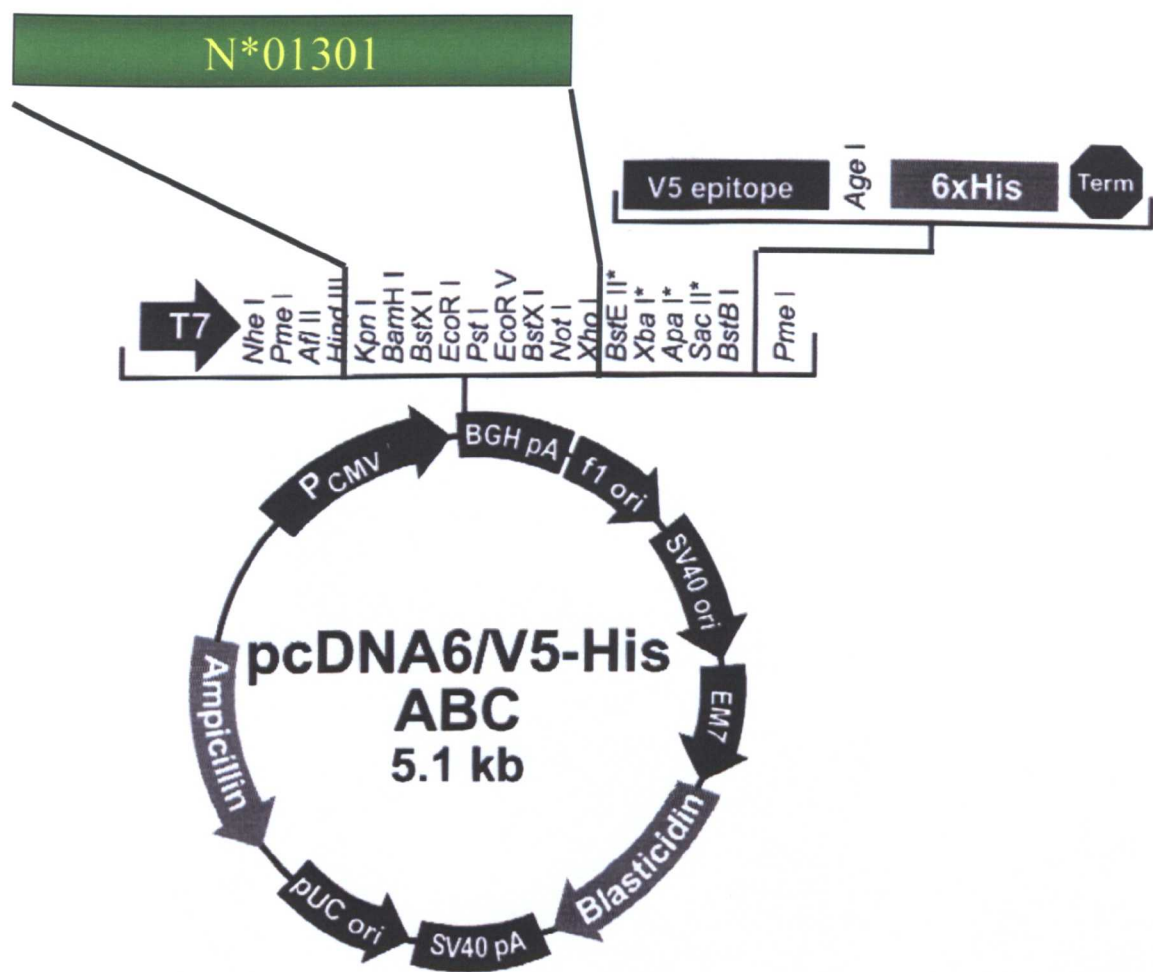
pcDNA3.1(-)HA-1E5T



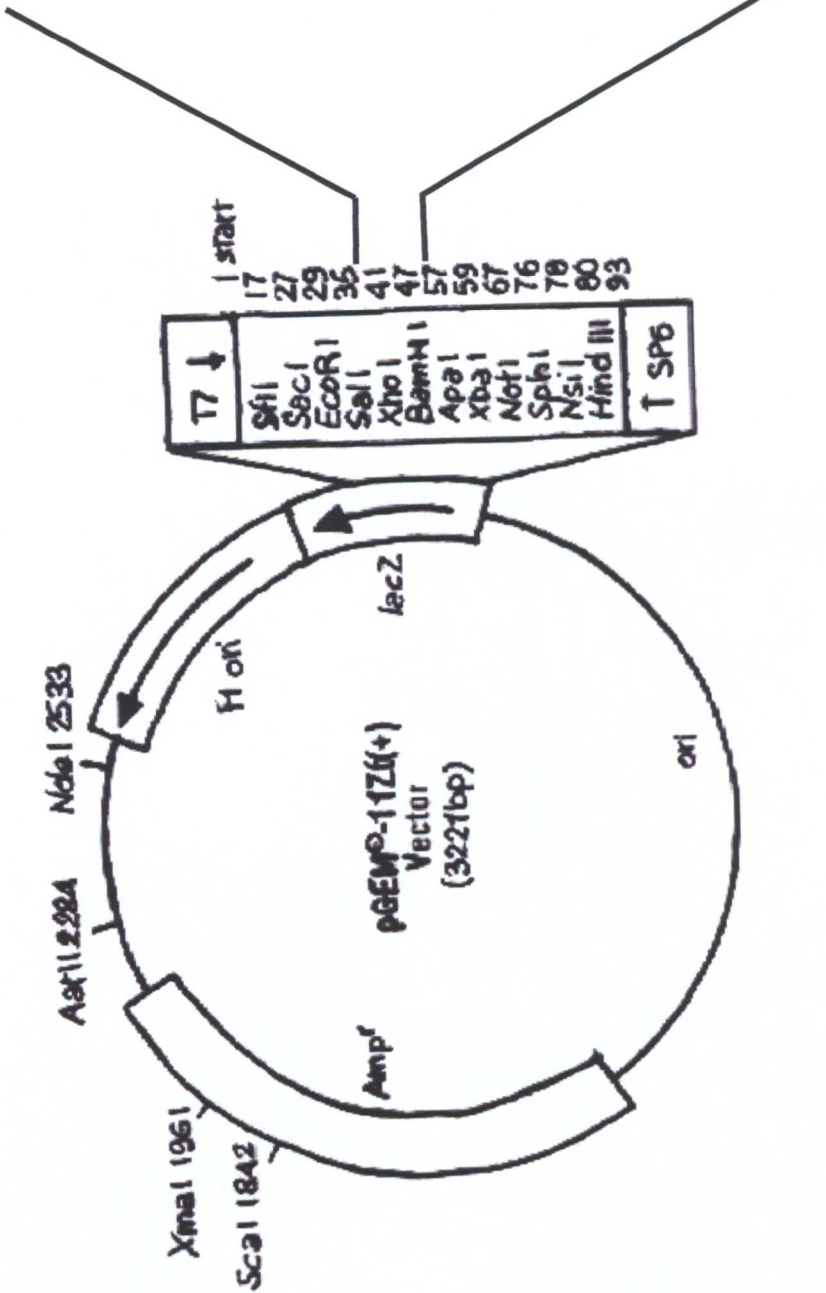
pcDNA6-N*50001



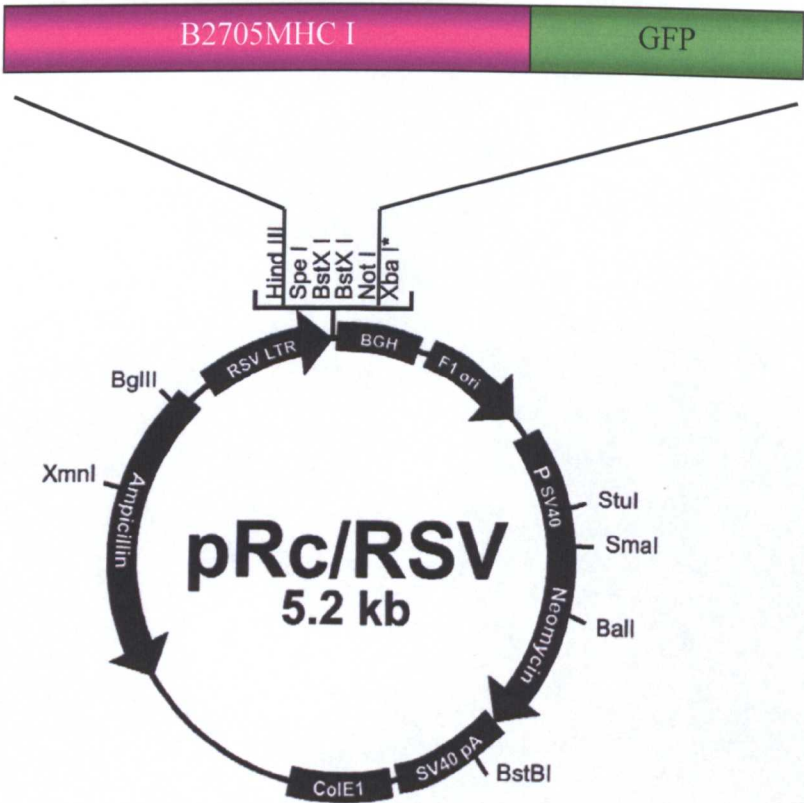
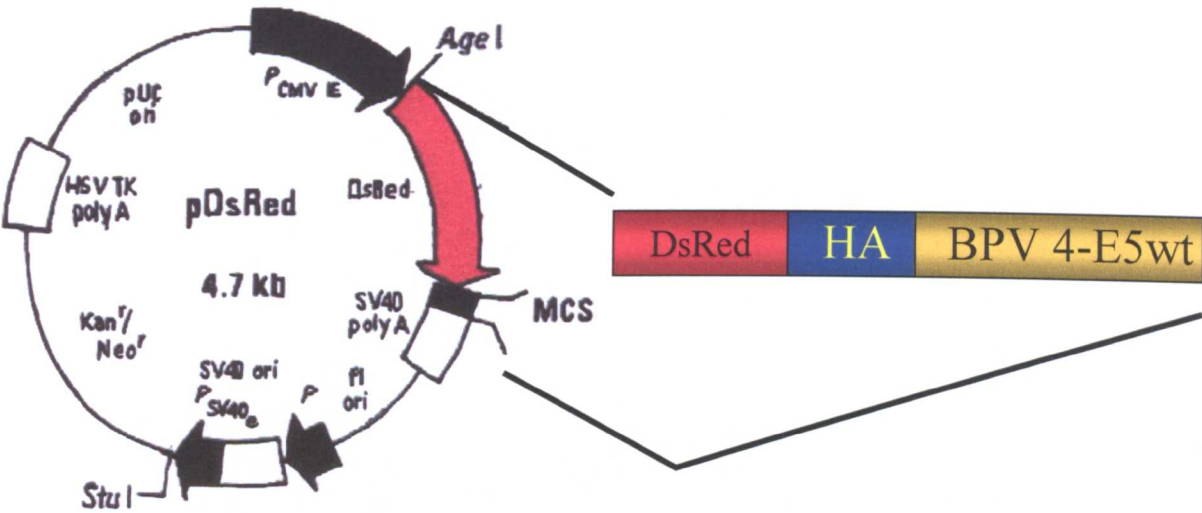
pcDNA6-N*01301-V5-His



pGEM-HLA-A1
pGEM-HLA-B8



pRFP-HA4E5
pGFP-MHC



2.1.12 Water

Distilled water for the preparation of buffer stocks was obtained from a Millipore MilliRQ 15 system, and for protein, enzyme, RNA or recombinant DNA procedures was further purified on a Millipore MilliQ System to 18M Ω /cm.

2.2 Methods

2.2.1 Molecular biology

2.2.1.1 DNA Extraction

DNA samples were purified by extraction with phenol:chloroform in order to remove contaminants, such as residual enzyme activities from a restriction reaction or detergent which might otherwise interfere with subsequent cloning steps. In the first round of extraction the DNA sample was mixed with an equal volume of phenol:chloroform. The aqueous DNA and organic phase were mixed thoroughly by vortexing, and then separated by centrifugation in a microcentrifuge at 14000 rpm for 5 minutes at room temperature. The upper aqueous phase was transferred in a clean eppendorf tube, care was taken not to transfer any of the interphase to the tube, and the extraction process repeated. The aqueous phase was then extracted with an equal volume of chloroform (chloroform:isoamyl alcohol, 24:1 v/v) by vortexing and centrifugation as described above. This was repeated to remove any traces of phenol from the aqueous phase. The aqueous phase was transferred to a fresh eppendorf for ethanol precipitation.

Ethanol precipitation was used to concentrate DNA samples and also to remove solute contaminants such as salt. The aqueous DNA solution was mixed with one-tenth volume of 3M sodium acetate pH 5.2 and 2-2.5 volumes of ice-cold ethanol. The sample was then mixed well by inversion several times and then stored at -20°C or, alternatively, placed on dry ice for 15-30 minutes to facilitate DNA precipitation. The precipitated DNA was collected by centrifugation in a microcentrifuge at 14000 rpm for 15 minutes at 4°C. The supernatant was discarded, the pellet was washed with 70 % ethanol to remove any trace of salt and dried under vacuum before resuspension in distilled water at an appropriate concentration. The DNA concentration was determined as described below.

2.2.1.2 Quantification of Nucleic Acids

The concentration of nucleic acid in a solution was determined spectrophotometrically in a WPA UV1101 Biotech spectrophotometer. Samples were diluted in dH₂O and transferred to a quartz cuvette with a pathway of 1cm. The spectrophotometer was initially calibrated using dH₂O only as a blank. The optical density readings were obtained at 260nm and 280nm; an O.D. reading of 1 at 260nm ($A_{260} = 1$) corresponds approximately to a concentration of 50µg/ml of double stranded DNA, for oligonucleotides an A_{260} of 1 was taken to correspond to ~35µg/ml, and for RNA an A_{260} of 1 was taken to correspond to 40µg/ml. The ratio between readings at 260nm and 280nm ($A_{260}:A_{280}$) provided an estimate of the sample purity; a ratio of ~1.8 indicated that preparations contained essentially no protein or phenol contamination.

2.2.1.3 Restriction Enzyme Digestion of DNA

Restriction digestions were carried out in small reaction volumes using enzymes and their appropriate concentrated buffer solutions according to the manufacturer's instructions. Plasmid DNA was incubated with 5-10 units enzyme/ μg DNA in a buffered solution ensuring that the total volume of enzyme added did not exceed one tenth of the final reaction volume. Small quantities of plasmid DNA ($<5\mu\text{g}$) were routinely digested in a $20\mu\text{l}$ reaction volume as specified by the manufacturer for 1 hour at 37°C . Large digests were carried out in proportionally larger reaction volumes. The digestion fragments were analysed by agarose gel electrophoresis as described below.

2.2.1.4 Agarose Gel Electrophoresis

In general, 1% (w/v) agarose gels were used, but smaller fragments (100-400) were separated on 2-4% gels. Low melting point agarose was used at a concentration of 1% (w/v) in order to isolate and purify required DNA restriction fragments. Gel mixes containing the appropriate amount of agarose were dissolved in $1 \times$ TBE buffer by heating the solution in a glass conical flask in a microwave oven until all the particles of agarose gel had dissolved. $0.5\mu\text{g/ml}$ ethidium bromide was added to the melted agarose when the agarose was hand hot then the gel was poured with the addition of a comb with the required number and size of teeth placed immediately into the gel to form the sample wells. The gel was submerged in $1 \times$ TBE buffer. The samples containing $1 \times$ loading buffer were loaded in each well along with an appropriate size marker (e.g. 100bp ladder, 1Kb ladder) into the first and/or last well in the gel and run at 70-100 constant voltage usually until the samples' blue dye front was 1-3 cm from the end of the gel. The separated DNA was visualised by

illumination with short wave (312nm) UV light and photographed through a red filter onto video print paper using an UVP gel documentation system.

2.2.1.5 Isolation and Purification of DNA Restriction Fragments from Agarose Gels

The DNA fragment to be used for cloning was recovered from low melting point agarose gels and visualised as described in section 2.2.1.4. The fragment was cut out of the gel with a clean scalpel blade and the gel slice placed in an eppendorf tube. Extraction of the DNA fragment from the agarose was achieved using a Qiagen Qiaquick gel extraction kit following the manufacturer's instructions.

2.2.1.6 Ligation of DNA Fragments

Both vector DNA and the DNA fragment to be inserted into the vector were separately digested with restriction enzymes and purified as described above and then isolated by gel electrophoresis as described in section 2.2.1.4. The vector DNA was dephosphorylated at its termini to prevent re-ligation. After the vector DNA had been linearized by digestion, the reaction mixture was adjusted by adding dephosphorylation buffer and 1 unit of Calf Intestinal Alkaline Phosphatase (CIP) was added to the reaction mixture and incubated at 37°C for 1 hour. The reaction was stopped by heating to 75°C for a further 15 minutes. All enzyme activity in the reaction was finally stopped by heating to 90°C for a further 5 minutes.

The DNA fragment was purified using a Qiagen Qiaquick purification kit following the manufacturer's instructions and then resuspended in appropriate volume of distilled water and stored at -20°C. The DNA fragment was incubated with dephosphorylated vector (100pg) at a ratio of 1:1; 1:3; 3:1 respectively in a reaction

containing 1 × ligase buffer and 1 unit of T4 ligase at 16°C overnight always carrying alongside a background control reaction containing the vector alone. Dilutions of this reaction volume were used to transform competent bacterial cells (section 2.2.1.7).

2.2.1.7 Transformation of *E. coli* DH5α or XL1-blue cells

Plasmids were propagated in commercially available *E. coli* DH5α or XL1-blue competent cells supplied as frozen stocks (Invitrogen and Stratagene, respectively) kept at -70°C until use. Bacteria were transformed following manufacturer's instructions. Competent cells were thawed slowly on ice, and 20µl of aliquots put into prechilled 1.5ml eppendorf tubes. 1-2 ng of the appropriate plasmid DNA was added to each aliquot and mixed by gently moving the pipette tip through the cells while dispensing. The cells were then incubated on ice for 30 minutes before being heat shocked for 45 seconds at 42°C. The tube was then immediately placed on ice for 2 minutes. 180 µl of room temperature SOC Media (2% Bactotryptone, 0.3% Yeast Extract, 10mM NaCl, 2.5mM KCl, 20mM Mg²⁺ Stock (equimolar ratio of MgCl₂·6H₂O & MgSO₄·7H₂O) and 20mM Glucose) was then added to each transformation reaction. The tube was then transferred to a shaking 37°C incubator (approximately 225rpm) for 1 hour to allow expression of the antibiotic resistant marker.

2.2.1.8 Glycerol Stocks

Host strains, and their derivatives containing useful plasmids, were stored as glycerol stocks for future retrieval. 850 µl of an overnight culture was mixed gently with 150 µl sterile glycerol in a 1.5 ml Nunc Cryotubes and stored at -70°C. A sterile plastic loop was used to retrieve an aliquot of cells as and when required.

2.2.1.9 Small Scale Preparation of Plasmid DNA (Miniprep)

Small amounts of plasmid DNA were extracted from transformed bacterial colonies to identify correct clones. Single colonies of bacteria carrying the required plasmid were picked using a sterile yellow pipette tip and grown in 5 ml culture of L-Broth (1% w/v Bactotryptone, 0.5% w/v yeast extract, 1% w/v NaCl) containing antibiotic (100µg/ml Ampicillin or 50µg/ml Kanamycin) at 37°C in a shaking incubator (225rpm) overnight. 10 separate colonies were generally picked for screening at any one time. Bacteria were pelleted from 1.5ml of overnight culture by spinning in a microcentrifuge (14000rpm) for 30 seconds at room temperature. DNA was prepared using the QIA prep Spin plasmid miniprep kit following the manufacturer's instructions.

2.2.1.10 Large Scale Preparation of Plasmid DNA (Maxiprep)

Bacteria containing the plasmid of interest were streaked onto an L-agar plate containing the appropriate antibiotic and the plate inverted and incubated overnight at 37°C to allow colony formation. A single colony was picked, using a sterile yellow tip, from this plate and used to inoculate a sterile universal tube containing 5 ml of L-Broth medium and the appropriate antibiotic (i.e.: 100µg/ml Ampicillin) which was then put in a shaking incubator at 225rpm overnight at 37°C. This culture was then added to 500 ml of broth, containing 100µg/ml Ampicillin in a 1 litre glass conical flask (to allow good aeration), then returned to the shaking incubator for 24 hours. DNA was prepared using the QIA prep Spin plasmid maxiprep kit following the manufacture's instructions.

2.2.2 Cell Culture and Transfection

2.2.2.1 Cell Culture

All cell culture work was performed following strict aseptic techniques inside a laminar flow hood (Class II Microbiological safety Cabins; Gelaire BSB4). Cells were incubated in dry 37°C incubators containing 5% (v/v) CO₂ (Napco Model 5410 Genetic Research Instrumentation LTD).

2.2.2.1.2 Primary bovine fibroblasts (PalF)

Primary bovine fibroblasts (PalF) were isolated from the palate of bovine foetuses of less than 5 months gestation obtained from the post-mortem room at the Glasgow University Veterinary School, grown and used at passages 2-8 (Jaggar *et al.*, 1990). Cells were routinely cultured in DMEM containing 10% foetal calf serum, 37.5 µg/ml penicillin, 50 µg/ml streptomycin and subcultured to maintain subconfluent monolayers. Cells were expanded and stocks were made and frozen down in liquid nitrogen for further experiments.

2.2.2.1.3 Stable transfection of PalF cells

PalF cells were transfected with a range of plasmids (see below) using a cationic lipid (DOTAP, Boehringer Mannheim) and were selected in medium containing 500µg/ml G418 for 21-28 days, and G418-resistant colonies were then picked and expanded. All transfectants were routinely maintained in DMEM supplemented with 500µg/ml G418 (Pennie *et al.*, 1993; O'Brien *et al.*, 1999; Ashrafi *et al.*, 2002). The plasmids used were:

- pZipneoHA4-E5, encoding G418 resistance and carrying the BPV-4 E5 ORF containing a sequence encoding the 11 residue HA epitope of the influenza virus, 5' to the 4-E5 sequence, has been reported previously (Faccini *et al.*, 1996; O'Brien *et al.*, 1999). pZipneoHA4-E5 served as the template for mutant construction. 4-E5 mutants containing single amino acid substitutions at residue 17 (N17A and N17Y), a C-terminus truncated form of 4-E5 (4-E5T), in which a premature stop codon has been introduced at residue 33 and a C-terminus truncated form of BPV-1 E5 (1-E5T) were produced by site-direct mutagenesis as described in O'Brien *et al.*, 1999.
- pZipneoHA1-E5, encoding G418 resistance and carrying the BPV-1 E5 ORF containing a sequence encoding the 11 residue HA epitope of the influenza virus, 5' to the 1-E5 sequence, has been reported previously (Faccini *et al.*, 1996; O'Brien *et al.*, 1999).
- pZipneoE7, expressing the BPV-4 E7 ORF, has been described previously (Pennie *et al.*, 1993).
- pT24, a pUC13-derived plasmid containing the 6.6-kb activated human c-Ha-ras oncogene, has been described previously (Pennie *et al.*, 1993).
- pJ4 Ω 16-E6 was a gift from Dr. L. Crawford (Department of Pathology, University of Cambridge). This plasmid construct is a pBR322 derivative containing the HPV-16 E6 ORF and has been described previously (Pennie *et al.*, 1993).

In these cells, the combination of BPV-4 E7 (pZipneoE7), E5 from BPV-4 (pZipneoHA4-E5) or BPV-1 (pZipneoHA1-E5), and activated *ras* (pT24) induced cell transformation but immortalization was conferred by HPV-16 E6 (pJ4 Ω 16-E6)

(Pennie *et al.*, 1993), as the BPV-4 genome does not encode an E6 protein (Jackson *et al.*, 1991).

Normal cells were designated PalF or “parental”; cells transformed by HPV-16 E6, BPV-4 E7 and activated *ras* were designated “no E5”, cells transformed as above but with the addition of either BPV-1 or BPV-4 E5 protein were designated “1-E5 or 4-E5”, respectively. Cells were expanded and stocks were made and frozen down in liquid nitrogen for further experiments.

2.2.2.2 Maintenance of cells in culture

Cells were fed twice weekly, old medium was aspirated from sub-confluent flasks and fresh medium added. Replating was performed as follows: for T175cm² tissue culture flask medium was aspirated off and the cells washed once with 10 ml phosphate-buffered saline (PBS). The PBS was removed and 4 ml of 1x trypsin solution (in Hanks Balanced Salt Solution (HBSS) w/o Ca and Mg w/EDTA 4NA [Ethylene Diamine Tetra Acetic Acid Trisodium Salt]), which had been pre-warmed to 37°C, was added to cells. Flasks were transferred to the 37°C incubator until the cells had detached from the flasks. Complete medium was added and the cell suspension transferred to a sterile universal tube. The cells were pelleted by centrifugation at 1000rpm for 5 minutes at room temperature. The pellet was then resuspended in fresh growth medium and the cells reseeded at a 1:10 density.

2.2.2.3 Long Term Cell Storage

To freeze cell stocks for storage, confluent cultures were trypsinised, and pelleted as described above (section 2.2.2.2). The pellet was then resuspended at a concentration of approximately 10⁶ cells/ml in growth medium containing 10% (v/v) DMSO. The

DMSO in the medium acts as a cryoprotectant but all solution must be chilled, as DMSO is toxic to cells at room temperature. Suspensions were divided into 1 ml aliquots in 1-2 ml Nunc cryotubes and placed in a polystyrene box and frozen, well insulated, at -70°C overnight to ensure a slow rate of cooling. The ampoules were then transferred to a liquid nitrogen bank containing labelled storage rack until required. Frozen stocks were recovered by removing the ampoules from liquid nitrogen and placing them into a small, covered bucket of water at 37°C. Once thawed, the cells were added to 10ml of the appropriate pre-warmed growth medium, centrifuged, resuspended in fresh growth medium and transferred to 175 cm² flasks.

2.2.2.4 Transient Transfection for Immunofluorescence

Cells were seeded into 24-well plates containing coverslips at a density of 10⁴ cells/well and grown overnight to approximately 80% confluence. Plasmids (0.6 µg) were transfected into cells using LIPOFECTAMINE and PLUSTM Reagent (Invitrogen/Life Technologies, UK) according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were washed twice with PBS and then fixed in fixing solution (19ml PBS, 1ml 37% formaldehyde, 0.4g sucrose) for 10 min at room temperature. Following a further three washes with PBS, the coverslips were removed from the well and mounted in AF1 (Citifluor, UK) and analysed either using a Leica DMLB microscope or a Zeiss LSM 510 microscope.

2.2.5 Transient transfection and luciferase transcription assay

10⁵ PalF cells were plated in each well of 6 well plates, in duplicate, with 5 ml of DMEM, 10% FCS at 37°C in 5% CO₂. Twenty four hours later, the cells were transiently co-transfected with 1µg of pBoLa-Luc and 1µg of pZipneo or 1µg of pZip-

4E5 (O'Brien *et al.* 1999), using the standard Lipofectamine Plus (Invitrogen, UK) method. After 3 hr, cells were washed twice with 2 ml PBS and incubated in medium for further 48 hr, with or without 250U/ml γ IFN, before being harvested and lysed in reporter lysis buffer. The lysates were either assayed for reporter enzyme activity immediately or stored at -20°C.

Luciferase activity was determined using a luminometer with automatic injection (Luminoskan Ascent-Thermo Labsystems). For each sample, 80 μ l of lysate and 80 μ l of Luciferase assay buffer (Promega) were used. Luciferase activity was normalised for protein content determined using the BCA assay (Sigma) (as described in section 2.2.4.2).

2.2.3 DNA and RNA analysis

2.2.3.1 Total RNA Extraction from Cell Lines

Cells were grown in a 175cm² (T175) flask to approximately 80% confluency. Total RNA was then extracted by use of the RNeasy Kit (Qiagen). The cells were washed once with pre-warmed sterile PBS and trypsinised and counted. 1x10⁷ cells of all cell lines were subjected to the RNA extraction method as per manufacturer's details. For all RNA extractions the concentration of RNA was measured spectrophotometrically as described in section 2.2.1.2. RNA samples were aliquoted and stored at -70°C.

2.2.3.1.1 Detection of MHC class I HC RNA by quantitative RT-PCR

Total RNA was extracted from Palf, no-E5 and PalF-4E5 cells using the RNeasy Mini kit (Qiagen, Sussex, UK), and residual DNA was removed by DNase I treatment according to the manufacturer's guidelines (Invitrogen, UK).

Real-time RT-PCR for MHC I heavy chain and bovine actin mRNA was carried out using the Taqman EZ RT-PCR kit (Applied Biosystems, Foster City, CA, USA) with gene-specific primers and FAM/TAMRA probe designed by primer express v1.7 software. 100 ng RNA was used per each reaction, done in triplicate. The primers, spanning exons 2 and 3, were:

BovMHC_Taq_Forward 5'-TCCGGGCGAACCTGAAC-3' and

BovMHC_Taq_Reverse 5'-ACATCTCCTGGAAGGTGTGAGAC-3' and the probe was 5'-CCGCACTCGGCTACTACAACCAGAGC-3'. Bovine actin primers were

BovACT_Taq_Forward 5'-CCTCACGGAACGTGGTTACAG-3' and

BovACT_Taq_Reverse 5'-TCTCCTTGATGTCACGCACAA-3' and the probe was 5'-TTACCACCACAGCCGAGCGGG-3'. RT-PCR was performed using an ABI

prism 7700 sequence detector. In each experiment, additional reactions with 10 ten-fold serial dilutions of template DNA were performed with each set of primers and probes on the same 96-well plates to generate standard curves. All samples were amplified in triplicate. The relative amounts of MHC I heavy chain and bovine actin mRNA were determined by using the standard curves.

2.2.3.1.2 Detection of E5 RNA in PalF cells by RT-PCR

RNA was prepared (see 2.2.3.1) and used as template for reverse transcription and PCR amplification by using the Perkin-Elmer Cetus RNA PCR kit. Primer sequences are as follows: BPV-4 E5 forward 5'-CCA TAC GAT GTT CCA GAT TAC GCT -3' and BPV-4 E5 reverse 5'-CCA TCC ATC TAA CCG AGT AAT AGT -3'

The reaction was carried out according to the manufacturers instructions, to the following final concentrations: 5mM MgCl₂, 1xPCR kit buffer II (500mM KCl, 100mM Tris-HCl), 1mM of each of dATP, dGTP, dTTT, dCTP, 1 unit RNase

inhibitor, 1µg RNA, 2.5 units MuLV reverse transcriptase, 0.15 µM of BPV-4 E5 reverse primer and DEPC-treated water to a final volume of 20µl. All samples were placed in the thermocycler and further incubated at 22°C for 10 minutes, 42°C for 15 minutes, 99°C for 5 minutes, and soaked at 4°C for 5 minutes. The above reaction volume was increased to 100µl by adding 2mM MgCl₂, 1x kit PCR buffer II, 0.15µM of BPV-4 E5 forward primer and 2.5 units of *Taq* Polymerase. Amplification proceeded for 35 cycles of 94°C for 30 seconds, 60°C for 1 minute, 70°C for 45 seconds and extension at 72°C for 10 minutes using the Perkin-Elmer Cetus 9600 thermocycler. 5 µl of each sample containing 1x loading buffer was then analysed by agarose gel electrophoresis to ensure correct amplification. (Ashrafi 1998).

2.2.3.1.3 Detection of BPV-4 E5 RNA by quantitative RT-PCR

RNA was extracted from PalF-4E5 and PalF-N17A cells using RNAeasy mini Kit (Qiagen, Sussex, UK) and residual DNA was inactivated using DNase I treatment (Invitrogen). Q-RT-PCR for BPV-4 E5 and bovine β-actin mRNA was carried out using the Taqman EZ RT-PCR Kit (Applied Biosystems, Foster City, CA). Each reaction was performed in triplicate using 100ng of RNA. Oligonucleotide primers, designed using Primer Express (v 1.7, Perkin Elmer, Oak Brook, IL), were as follows:

BPV4 E5 F5'-TGTCTTTGTGGCTTATCTATGTTTTGT-3';

BPV-4 E5 R5'-CCGAGTAATAGTAGAAATTAACAGAAGGTACAC-3'; and

FAM/ TAMRA probe 5'-CTTTTCTGGTGTGCTTTTAATTTTCTTGCACTGTTA-3'.

BovACT_Taq_Forward 5'-CCTCACGGAACGTGGTTACAG-3' and

BovACT_Taq_Reverse 5'-TCTCCTTGATGTCACGCACAA-3' and the probe was 5'-TTACCACCACAGCCGAGCGGG-3'.

PCR reactions were performed using an ABI prism 7700 sequencer. Standard curves were generated using 10-fold serial dilutions of each template DNA, which were used for quantifying the relative levels of E5 and β -actin RNA.

2.2.3.2 DNA Sequencing

The sequence of all new plasmids was checked using Taq terminator sequencing on an Applied Biosystems Prism 3100 Genetic Analyzer DNA sequencer. The region to be sequenced first underwent PCR amplification. The sequencing reaction mix was prepared by adding 500ng of template DNA (~6 μ l of standard miniprep), 2 μ l 5x sequencing buffer, 3.2pmoles the appropriate primer, 4 μ l of BigDyeTM Terminator Ready Reaction Premix (Applied Biosystems) made up with distilled H₂O to a total reaction volume of 20 μ l in 200 μ l thin walled eppendorf tubes. The samples were placed in a MJ Research PTC-200 Peltier Thermal Cycler and exposed to 25 cycles of 96°C for 10seconds, 50°C for 5seconds and 60°C for 4 minutes. The PCR products were purified using PERFORMA[®] DTR Gel Filtration Cartridges. The columns were first spun at 3000rpm for 2 minutes to remove buffer. The columns were then transferred to sample collection tubes. The PCR sequencing reaction mix was then loaded onto the centre of the gel column. The column was spun for 3000rpm for 2 minutes. The sample was then dried under vacuum for 15-20 minutes and then resuspended in 20 μ l Hi-Di formamide (99.5% formamide, 0.11% EDTA, 0.39% water).

Table 2.2 Sequencing primers

Primer Name	Position	Primer Nucleotide Sequence
T7 forward 5'-3'	864-882	5' -ATTAATACGACTCACTATAGGGA-3'
BGH reverse 5'-3'	1039-1021	5' -CTAGAAGGCACAGTCGAGGC-3'
SP6 reverse 5'-3'	1017-998	5' -ATTTAGGTGACACTATAGAA-3'
T3 reverse 5'-3'	806-787	5' -ATTAACCCTCACTAAAGGGG-3'
M13 reverse 5'-3'	826-807	5' -GGAAACAGCTATGACCATG-3'

2.2.4 Protein Analysis

2.2.4.1 Protein Preparations from Cells for Western Blot Analysis

Cells were lysed by aspirating the culture medium off, washing the cell monolayer once with ice-cold PBS, the PBS was completely removed by aspiration.

200µl SDS-lysis buffer (100mM Tris-HCl pH6.8, 2% SDS, 2% glycerol and one tablet protease inhibitor cocktail in 10ml final volume) was added to the 100mm/plates and cell debris was scraped off and transferred to microcentrifuge tubes. The cells were further disrupted by repeated aspiration with a syringe. The cell debris was pelleted at 14000 rpm and the supernatant transferred to a new microcentrifuge tube.

2.2.4.2 Protein Concentration Assays

The BCA/CuSO4 Protein assay was used to spectrometrically determine the protein concentration of dilute solutions following the manufacturer’s instructions. Proteins reduce alkaline Cu(II) to Cu(I) in a concentration-dependent manner. Bicinchoninic acid is a highly specific chromogenic reagent for Cu(I), forming a purple complex

with an absorbance maximum at 562nm. 10µl of protein solution was placed in separate wells in 96 well plate. 200µl of developing solution (5ml BCA (Biocinchoninic acid) solution plus 100µl of 4% (w/v) CuSO₄ (copper II sulphate pentahydrate solution) was added to the protein samples and incubated at 37°C for 30 minutes.

The absorbance of each sample was read at 562nm using a WPA UV1101 Biotech photometer plate reader. The absorbance reading was converted to concentration in µg/ml for each sample using a standard curve generated from a series of control BSA solutions of known concentration. The actual concentration of each protein sample was calculated after multiplying by the relevant dilution factor.

2.2.4.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were resolved according to the molecular weight using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). All Western blot analysis was performed using NuPAGE™ pre cast gel system unless otherwise stated. 10µg of each protein sample were electrophoresed on NuPAGE™ 4-12% Bis-Tris gel (Invitrogen, UK) under denaturing conditions. Prior to loading, equivalent amount of each protein sample (10µg) was mixed with 5µl of 4x SDS gel loading buffer (Invitrogen, UK) and 2µl of 2x reducing agent (Invitrogen, UK) made up to 20µl with dH₂O. Samples were then boiled for 10minutes at 70°C. The prepared protein samples were then loaded into consecutive wells and 10µl See Blue ® Plus 2 Pre-Stained protein molecular weight marker (molecular weight range 3KD-250KD depending on running buffer used) added to the first and/or last well on the gel. The gel was run by electrophoresis at a constant voltage of 200V for 45minutes. The running buffer (Nu

PAGE™ MES buffer, Invitrogen, UK) was used with 500µl antioxidant (Invitrogen, UK) added to 200ml of the running buffer for the inner tank. Once the dye front was near at the bottom of the gel, the gel was removed and used for western blot analysis.

2.2.4.4 Western Blotting

Separated protein samples were transferred to a nitrocellulose membrane using the NuPAGE™ wet blotting tank. For this purpose the gel was removed from the electrophoresis tank and transferred onto a nitrocellulose membrane (Invitrogen, UK). Before transferring the membrane, the NuPAGE™ gel sandwich was pre-soaked with transfer buffer with 500µl antioxidant added to 1L of transfer buffer (with 10% or 20% methanol depending on the number of gels being transferred). The gel sandwich contains a pre-cut nitrocellulose membrane sandwiched between two filter papers. The gel tank was set up as follows:-

1. The first sheet of filter paper, soaked in transfer buffer was placed neatly on to the gel avoiding any air bubbles.
2. Then the nitrocellulose membrane was laid on the gel, and then a further sheet of filter paper, all soaked in transfer buffer was added to the top.
3. The sandwich was rolled with a glass pipette to eliminate any air bubbles
4. The sandwich was placed into the transfer tank and run at 30V for approximately 60 minutes, the time taken for the pre-stained marker proteins to be completely transferred.

Once the transfer was completed, the membrane was blocked by shaking for a minimum of 1 hour in 50 ml of block buffer (5% Marvel [dried milk] in TBS-0.5% Tween) at room temperature. The nitrocellulose filter was washed in wash buffer

(TBS-T) for 10 minutes. The filter was then placed in 10ml blocking buffer containing suitable primary antibody and incubated at room temperature for 1 hour (to overnight depending on the antibody used) with gentle shaking. The primary antibody solution was removed and the filter rinsed in blocking buffer then washed 1x 15 minutes and 2x 5minutes in 100ml volumes of fresh TBS-T. The filter was then incubated in 20 ml blocking buffer containing a 1/2000 or 1/5000 dilution of the applicable HRP-linked secondary antibody for 1 hour at room temperature with gentle shaking. The filter was washed 1x for 15 minutes and 4x for 5 minutes with TBS-T buffer. Excess surface liquid was removed from the filter by briefly blotting with a piece of Whatman 3MM paper. The detection consisted of incubating the filter in an equal volume of an Amersham Enhanced chemilluminescence (ECL) detection reagents 1 and 2 for 1 minute at room temperature. The excess detection solution was drained off the nitrocellulose filter and this was then wrapped in Saran wrap and exposed to Amersham ECL film for 30 seconds and up to 30 minutes (depending on the strength of the signal).

2.2.4.5 Western Blotting of IFN, inhibitors and monensin treated cells

PalF and E5-transformed cells were seeded in 24-well plate and treated or untreated with 500 (or 250)U/ml ovine recombinant γ -IFN for 48 h and treated with a panel of lysosome and proteasome inhibitors :1 μ M Bafilomycin A1, 50 μ M LY 294002, 40mM NH₄Cl for 24 h, and 5 μ M MG-132 and 100 μ M ALLN for 8 h at 37⁰C in 5% CO₂. Ovine recombinant γ -IFN and 2D6, a control supernatant from cells not expressing IFN were a kind gifts of Dr G Entrican (Moredun Research Institute, Penicuik, UK). For monensin treatment the cells were seeded in T75 flasks and were treated with 25 μ M monensin for 30 min, 3 or 12 h.

Cells were removed from 24-well plate or from the flasks by trypsinization, washed with PBS, then lysed in lysis buffer: 100 mM Tris HCl, pH 7.5, 2% SDS, 2% glycerol in presence of protease inhibitors tablets and insoluble material was removed by centrifugation at 14.000rpm. Ten μ g of lysate were electrophoresed in 4 - 12% NuPAGE gels, and proteins transferred to nitrocellulose membrane (Invitrogen) using a blotting apparatus at 30V for 1 h. The membranes were blocked in 5% milk/TBS/Tween 20 (0.5%) at room temperature for 1 h before incubation with 1:100 dilution of mAb IL-A88 raised against the MHC I heavy chain, or 1:20.000 dilution of mAb AB-1 specific for actin. After repeated washing with TBS/Tween 20 (0.5%) the membranes were incubated with 1:2000 dilution of anti-mouse Ig-HRP for mAb IL-A88, and 1:5000 dilution of anti-mouse IgM-HRP for mAb AB-1, in 5% milk/TBS/Tween 20 (0.5%) for 1 h at room temperature. The membranes were washed three times with TBS/Tween 20 (0.5%) and bound antibody was detected by enhanced chemoluminescence staining (ECL).

2.2.4.6 Stripping Western Blot Membranes

If a western blot membrane was required for multiple analyses with different probe antibodies then the initial primary and secondary antibodies were removed from the membrane by stripping. The membrane post exposure to the Amersham ECL film was washed for 10 minutes three times in PBS before being incubated for 30 min at 50°C in Stripping Buffer (100mM 2-Mercaptoethanol, 2% SDS, 62.5mM Tris HCl pH 6.8). The membrane was then washed with copious amounts of PBS until all traces of the SDS were safely removed. The membrane was then blocked as described in 2.2.4.4 for the next probing primary antibody.

2.2.4.7 *In vitro* Transcription Translation and Immunoprecipitation

In vitro transcription/translation reaction was performed using the TNT® T7 Quick Coupled Transcription/Translation System (Promega, UK) in presence of Redivue L-[³⁵S] Methionine (Amersham Pharmacia Biotech, UK) following the manufacturer's instructions. Briefly, 1 µg of plasmid DNA was mixed in a 50µl reaction containing TNT® mix (TNT® lysate with energy generating system, T7 RNA polymerase, nucleotides, salts, recombinant RNasin® ribonuclease inhibitors) in presence of canine microsomal membranes (CMM) (Promega, UK) at 30°C for 1.5 h.

Half of each transcription/translation reaction product was immunoprecipitated with either 10 µl rabbit antiserum Ab 274, raised against the C-terminus of the BPV-4 E5 protein (Anderson *et al.* 1997, Pennie *et al.* 1993; Araibi *et al.* 2004) or with 5 µl of mAb HA.11 (Cambridge Bioscience, UK) against the HA epitope tagging E5, or with 3 µl mAb IL-A88 raised against the bovine MHC I HC; 10 µl mAb HC10 specific for human HLA-A,-B,-C class I heavy chain; 1:2 dilution MAC 291 raised against the equine MHC class I (Animal Health Trust, Suffolk, UK); 1:10 dilution H58A raised against the equine MHC class I (VMRD Inc, USA).

The other half of each reaction was left without antibody as a negative control. For co-immunoprecipitation experiments the individual transcription/translation products were mixed in equivalent amounts and immunoprecipitated with double the amount of either antibodies. For competition experiments, 10µg of a synthetic peptide corresponding to the C-terminal 12 amino acid residues of E5 (Anderson *et al.* 1997; Araibi *et al.* 2004) or the transcribed/translated unlabelled product of the E5T mutants (BPV-1 E5 or BPV-4 E5) were added to labelled N*01301 for an hour before the addition of labelled E5 (either 1-E5 or 4-E5) as above. After incubation overnight at 4°C, protein G-sepharose bead suspension (Sigma, UK) was added for 1 h at 4°C.

Following two washes in a high salt buffer (50mM Tris HCl pH 7.5, 500mM NaCl, 1% NonidetP-40, 0.05% NaDoc) and one wash in a low salt buffer (50mM Tris HCl pH 7.5, 1% NonidetP-400, 0.05% NaDoc) the sepharose beads were resuspended in 20 μ l of SDS loading buffer, heated at 75°C for 10 min, and then were electrophoresed in 4-12% NuPAGE gels (Invitrogen) as described in section 2.2.4.3. Gels were fixed with glacial acetic acid and methanol, incubated for 15 min in AmplifyTM Fluorographic reagent (Amersham, UK), dried and exposed for autoradiography at -70°C overnight or exposed on a screen for quantification on a Storm 840 apparatus using a ImageQuant v5.2 software.

2.2.4.8 *In vivo* co-immunoprecipitation

Control PalF cells and PalF-4 E5 cells were treated with γ -IFN and MG132 as described in 2.2.4.5. Cells were lysed in RIPA buffer containing a cocktail of protease inhibitors (Roche, UK). Protein lysate (100 μ g) were immunoprecipitated with 1:10 dilution of rabbit antiserum Ab 274. Immunoprecipitate (10 μ g) were run in NuPAGE gels, transferred onto nitrocellulose membranes and immunoblotted with 1:1000 dilution of mAb IL-A88. A parallel gel was stained with Coomassie blue. Briefly, the gel was incubated in Coomassie staining solution (0.05%(w/v) Coomassie Brilliant Blue R-250, 40% (v/v) ethanol, 10% (v/v) glacial acetic acid, 50% (v/v) water) for 1h with gentle agitation and then the stained gel was incubated in destaining solution (40% (v/v) ethanol, 10% (v/v) glacial acetic acid, 50% (v/v) water) until the background became clear.

2.2.5 FACS analysis

2.2.5.1 Detection of MHC class I and transferrin receptor by FACS

Cells were grown in a 175 cm² flask to approximately 80% confluence. After removal of the medium, the cells were washed once with PBS, then detached from the flask with trypsin/EDTA and pelleted at 1000rpm at room temperature for 5 min. The cell pellet was resuspended in DMEM, 10% FCS, for 1 h at 37°C to allow surface antigens to be re-expressed. The cells were washed and re-suspended in PBS, 1% BSA (PBS-B) at 10⁷ cells/ml. For the detection of surface MHC I, 100 µl of cells were aliquoted and incubated with an equal volume of anti-bovine MHC class I monomorphic mAb IL-A19 for PalF cells, and anti-mouse H-2Ld mAb (CL9011-A, Cedarlane Laboratories) for NIH 3T3 cells, for 30 min at 4°C. For the detection of surface transferrin receptor, cells were incubated with mAb IL-A165 (Naessens and Davis 1996). The cells were washed three times in PBS-B, then gently resuspended in 100 µl of PBS-B and incubated with an equal volume of a 1:200 dilution of anti-mouse IgG-FITC (Sigma) at 4°C for 30 min in the dark. The cells were washed and resuspended in 500 µl PBS-B and analysed by flow cytometry. If the flow cytometry analysis was not carried out immediately, the cells were resuspended in 500 µl of 3% paraformaldehyde (PFA) in PBS and kept at 4°C. For the detection of intracellular MHC I, the cells were fixed in 3% PFA in PBS for 20 min at room temperature, washed in PBS-B and permeabilized with 0.5% saponin in PBS-B for 30 min at room temperature. Following a further wash in PBS-B, the permeabilized cells were stained with mAb IL-A19 as described above. Samples were examined in a Beckman Coulter EPICS Elite analyser equipped with an ion argon laser with 15 mV of excitation at 488 nm. The data were analysed using Expo 2 software.

2.2.6 Immunofluorescence

2.2.6.1 Visualization of Golgi apparatus by direct immunofluorescence

The Golgi apparatus was visualized by staining with BODIPY-TR-ceramide. Cells were grown until 80% confluent in single well chamber slides. After removal of medium cells were washed twice with serum free DMEM, 25 mM HEPES (DMEM-H) and incubated in 200 μ l of 5 μ M BODIPY-TR-ceramide, which localizes to the GA, in DMEM-H for 30 min at 4°C. Cells were then washed with PBS and incubated in DMEM-H for 30 min at 37°C. After removal of the medium and washing with PBS, cells were fixed with fresh 3% PFA for 20 min at room temperature and mounted with Vectashield (Vector Laboratories Inc, UK).

2.2.6.2 Visualization of Golgi apparatus and endogenous MHC I by indirect immunofluorescence

Cells were grown until 80% confluent in single well chamber slides. The cells were washed twice with PBS and fixed with fresh 3% PFA in PBS for 20 min at room temperature. After the PFA fixation, a second fixation was performed by dipping the chamber slides in methanol for 4 min; then the cells were washed three times in PBS. For GA detection, the cells were incubated with mAb 4A3 for 30 min at room temperature, washed as above and incubated with Alexa-Fluor™ 488 goat anti-mouse IgG(H+L) conjugate (Molecular Probes, Europe BV) for 30 min at room temperature. For the detection of endogenous surface MHC I, the cells were incubated with mAb IL-A19 or mAb IL-A88 for 1 h at room temperature and washed three times as above. The cells were then incubated with anti-mouse IgG-FITC (Sigma) at RT for 1 h in the dark. Following three final washes with PBS, the slides were mounted in Vectashield (Vector Laboratories Inc, UK). The images were collected

with a LEICA DMLB fluorescence microscope equipped with a SenSys 1400 camera. Data sets were processed using the QUIPSTM Smart Capture imaging software (Vysis, UK).

2.2.6.3 Immunofluorescence of transient transfected cells

Round coverslips were first washed in distilled water, air-dried and put into a beaker for autoclaving. The coverslips were placed in 24 well tissue culture plates and cells seeded 1×10^5 cells per well. All transfections for immunofluorescence studies were performed using LIPOFECTAMINETM according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were washed twice with PBS and fixed by 10 minute incubation at room temperature with a fixing solution (19ml PBS, 1ml 37% formaldehyde, 0.4g sucrose) and washed three times with PBS.

For direct immunofluorescence the cells on coverslip were mounted in AF1 Citifluor and direct analysed using a Leica TCS SP2 confocal scanner (Leica-microsystems, Heidelberg Germany) with a x63 oil immersion objective lens, NA 1.4. Data sets were processed using the LSM 510 software.

For the indirect immunofluorescence the cells on coverslip were permeabilised by 10 minute incubation at room temperature with 1% NP40 in PBS containing 20% sucrose and washed three times with 1% FCS in PBS and then incubated with the appropriate antibody for 1 h at room temperature, washed in 1% FCS/PBS and then incubated with an anti-mouse antibody conjugated either with Texas Red or with FITC (Sigma). Following further washes in 1% FCS/PBS, coverslips were mounted in AF1 Citifluor and analysed as above.

CHAPTER 3: The Bovine Papillomavirus type 4 E5 protein retains MHC class I molecules in the Golgi apparatus

Background: E5 downregulation of MHC class I

BPV-4 E5 is localized in the endomembrane compartment of the cell. The expression of the protein in primary bovine cells leads to profound changes in cell morphology, with extensive vacuolization (Ashrafi *et al.* 2000) affecting particularly the GA that appears grossly distorted, swollen and fragmented, changes that do not occur in cells that do not express E5.

Proper functioning of the Golgi apparatus is necessary for a number of cellular processes including protein transport to the cell surface. MHC class I molecules are synthesized in the ER, post-translationally modified in the ER and GA and transported to the plasma membrane (Cresswell *et al.* 1999). This process appears to be inhibited in cells expressing E5, which have very little, if any MHC I on their surface, as assessed by FACS analysis, and very little amount of total (intracellular plus surface) MHC I HC as assessed by immunoblotting (Ashrafi *et al.* 2002).

In this study Ashrafi *et al.*, used two cell lines: primary embryonic bovine cells (PalF) transformed by papillomavirus oncogenes, with or without E5 from BPV-1 or BPV-4 (see section 2.2.2.1.3), NIH 3T3 mouse fibroblasts transformed by BPV-4 E5 (3T3 4-E5) (Ashrafi *et al.*, 2002). In addition, PalF cells acutely infected with a recombinant retrovirus expressing E5 were also examined (Ashrafi *et al.*, 2002).

In contrast to non-transformed normal cells, or transformed cells expressing other papillomavirus proteins, cells expressing E5 do not express MHC I on their surface, but retain it intracellularly, independently of the presence of other viral or cellular oncogenes, or of whether the cells are long-term transformants or acutely infected. In

these study lower levels of MHC I mRNA transcripts were also detected in the E5 transformed cells. It is resonable to assume that there is at least a degree of specificity in the disturbance of protein traffic by E5 as the expression at the cell surface, and presumably the transport of another membrane glycoprotein, the transferrin receptor, is not affected. The conclusion from this study was that expression of E5 prevents expression of MHC I to the cell surface and causes its retention within the cell (Ashrafi *et al.* 2002).

3 PalF E5-expressing cells

Primary bovine fibroblasts (PalF) were isolated and grown in cultured medium (DMEM containing 10% serum) and used at passages 2-8 (Jaggar *et al.*, 1990). Cells were sub-cultured at appropriate intervals to maintain sub-confluent monolayers. PalF cells were transfected using a lipofection transfection technique (DOTAP) with a range of plasmid DNAs and were selected in medium containing 500µg/ml G418 (as described in section 2.2.2.1.3 and in Pennie *et al.*, 1993; O'Brien *et al.*, 1999; Ashrafi *et al.*, 2002),

In these cells, the combination of BPV-4 E7, E5 from BPV-1 or BPV-4, and activated *ras* induced cell transformation but immortalization was conferred by HPV-16 E6 (Pennie *et al.*, 1993), as the BPV-4 genome does not encode an E6 protein (Jackson *et al.*, 1991). Normal cells were designated PalF or “parental”; cells transformed by HPV-16 E6, BPV-4 E7 and activated *ras* were designated “no E5”, and cells transformed as above but with the addition of either BPV-1 E5 or BPV-4 E5 protein were designated “1-E5 or 4-E5”. Cells were expanded and stocks of either PalF, no E5 and 4-E5 cells were frozen down in liquid nitrogen for further experiments.

3.1 Detection of E5 in PalF cells

Given the difficulty of protein detection due to the small size and highly hydrophobicity of E5, its expression in PalF cells was firstly assessed by dot blot analysis using a mouse monoclonal antibody raised against the HA epitope from the influenza virus hemagglutinin tagged at the N-terminus of E5, described previously (O'Brien *et al.*, 1999). E5 expression in PalF cells was also assessed by RT-PCR of E5 RNA (see section 2.2.3.1.2 from Ashrafi 1998) or by quantitative RT-PCR, as described in this thesis (see section 2.2.3.1.3 and figure 4.2 C). Additionally, cells used for either FACS analysis, immunofluorescence staining or western blots were periodically checked for E5 expression either by RT-PCR or quantitative RT-PCR by Miss Emma Tsirimonaki or Dr. Rubina Ullah (previous workers in this laboratory).

3.2 MHC I is retained in the GA in E5-transformed cells

Given the abnormal morphology of the ER and GA in E5-expressing PalF cells, and given the importance of these structures in protein traffic, we investigated whether E5 expression would disrupt protein transport and in particular MHC I transport to the cell surface. Parental PalF and transformed control (no E5) cells were incubated with IL-A19, a monoclonal antibody recognizing a monomorphic determinant of the bovine MHC class I complex (Bensaid *et al.* 1989) and analysed by flow cytometry. Both cell lines displayed MHC I on their surface as shown by the increase in relative fluorescence intensity in the presence of mAb IL-A19 (Figure 3.2 A,C). In contrast, there was no shift in fluorescence in the transformed cells expressing E5 (Figure 3.2 E,G) indicating that these cells do not present MHC I on their surface. To investigate if this was due to a defect in MHC I transport to the cell surface, the cells were permeabilized with saponin before staining to allow the penetration of the antibody

inside the cells and therefore measuring the total MHC I content. The permeabilized parental and transformed control cells exhibited a similar staining profile, not different from that obtained for surface MHC I (Figure 3.2 B, D). In contrast, the FACS profiles for the permeabilized E5 cells showed a positive staining for MHC I (Figure 3.2 F, H), indicating that in these cells MHC I was retained intracellularly and was not transported to the plasma membrane. However, the intensity of fluorescence and the mean fluorescence of the E5 permeabilized cells were also noticeably reduced when compared to the parental or transformed control cells (Figures 3.2 F, H and 3.2 I), indicative of reduced levels of total MHC I in E5 cells. These data show that MHC I is consistently absent from the cell surface in cells transformed by BPV-1 E5 (1-E5) or BPV-4 E5 (4-E5) (Ashrafi *et al.* 2002). To ascertain the intracellular localization of MHC I in E5 cells, PalF, no-E5 and 1-E5 or 4-E5 cells were stained with either mAb IL-A19 or IL-A88 (Bensaid *et al.* 1989; Toye *et al.* 1990), which recognize β_2 -microglobulin-associated MHC I heavy chain or free heavy chain, respectively. In PalF and no-E5 cells, MHC I was detected on the cell surface and in a Golgi-like structure (Figure 3.2 J). In contrast in both 1-E5 and 4-E5 transformed cells, MHC I was detected only in abnormal structures (Figure 3.2 J), reminiscent of the misshapen GA in these cells (Ashrafi *et al.* 2002).

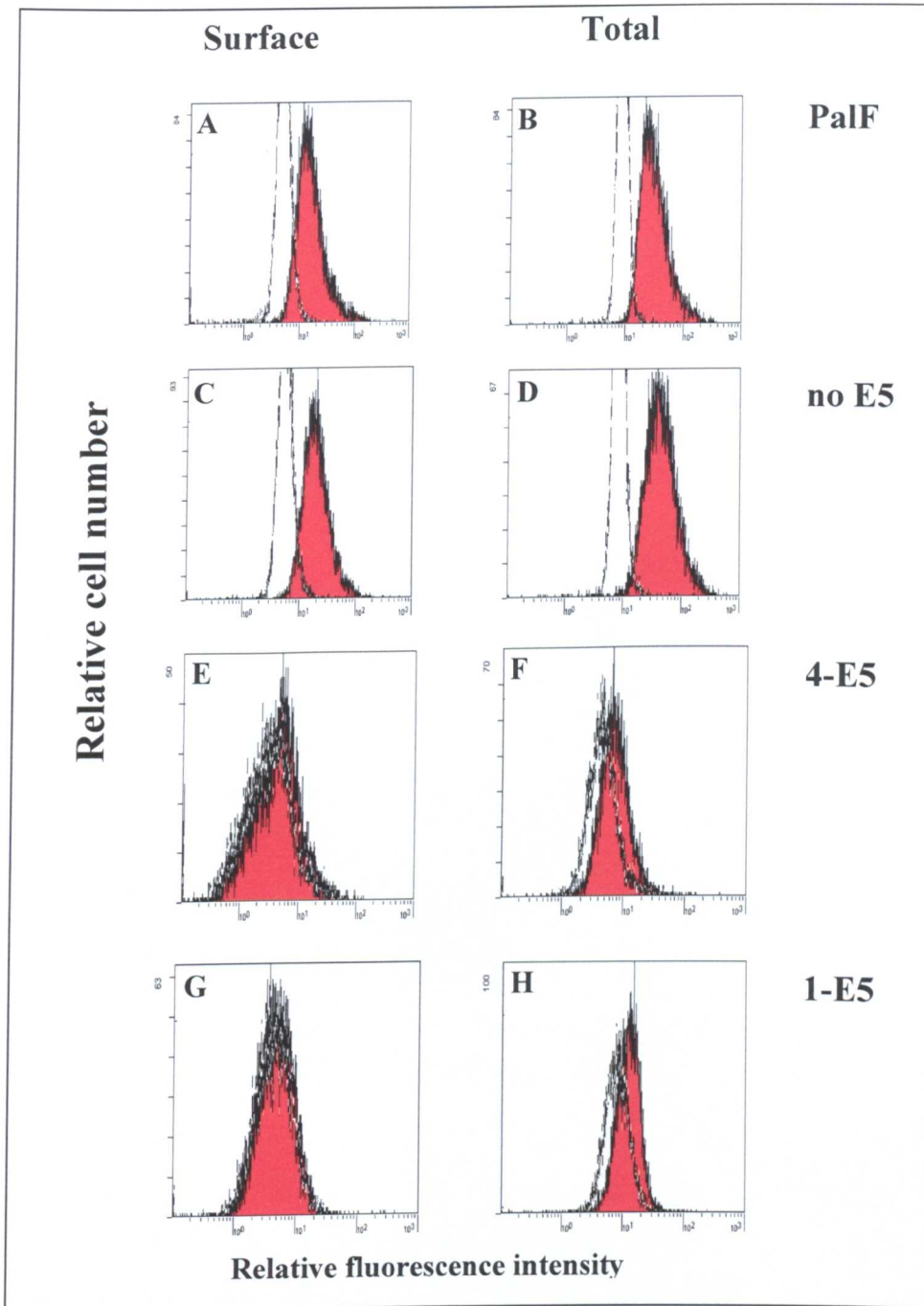


Figure 3.2 MHC I is absent from the cell surface of E5-transformed cells

FACS profile of MHC I expression in parental and transformed PalF cells.

PalF cells (A,B), transformed control cells (no E5) (C,D), 4-E5 transformed cells (E,F) and 1-E5 transformed cells (G,H) were stained with mAb IL-A19 and analysed by flow cytometry. The open histograms represent cells stained with FITC-conjugated secondary antibody only. Surface MHC I (solid histogram) was measured in intact cells (A,C,E,G) and total MHC I (solid histogram) was measured in saponin-permeabilized cells (B,D,F,H).

(experiment done by Dr. Hossein Ashrafi and published in Ashrafi *et al.*, 2002)

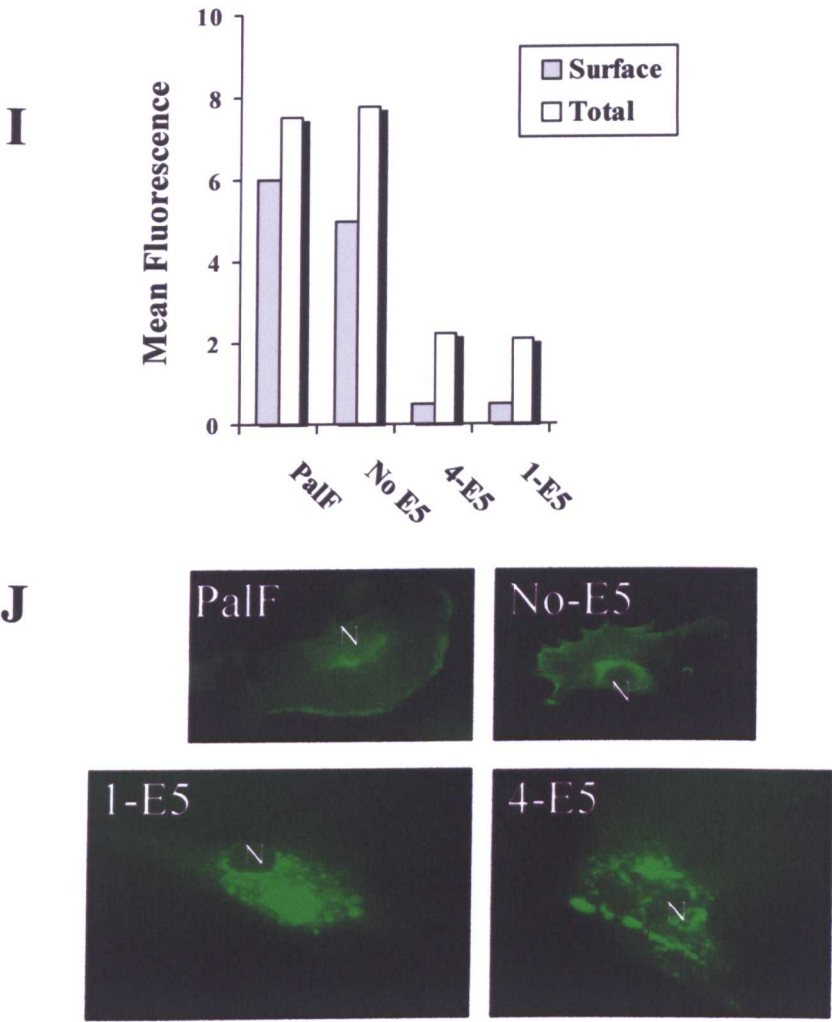


Figure 3.2 MHC I is absent from the cell surface of E5-transformed cells

I) Representative mean fluorescence for surface and total MHC I in transformed cells.

PalF, no-E5 and 1-E5 or 4-E5 cells were analysed for surface or total MHC I expression by FACS. The mean fluorescence was calculated from the flow cytometric analyses above (experiment done by Dr. Hossein Ashrafi).

J) Immunofluorescence detection of MHC I in PalF, no-E5, 1-E5 and 4-E5 cells. MHC I heavy chain was detected on the cell surface and in GA-like structures in parental and control cells, but only in GA-like structures in E5 cells. No fluorescence was detected in cells treated only with the secondary antibody. The images were collected with a LEICA DMLB fluorescence microscope equipped with a SenSys 1400 camera. Data sets were processed using the QUIPS™ Smart Capture imaging software (Vysis, UK). Magnification 40x. N=nucleus (experiment done by Dr. Hossein Ashrafi and representative figure from data published in Marchetti *et al.* 2002).

3.3 E5 and MHC I heavy chain colocalize in the Golgi apparatus

To define the intracellular structures in which MHC I was detected, PalF, no-E5 and 4-E5 cells were transfected with a plasmid encoding a fusion of green fluorescent protein (GFP) and the heavy chain of human MHC I B2705 (detail of construct in chapter 2, sections 2.1.10 and 2.1.11), and stained with the Golgi marker BODIPY-Texas Red-ceramide as described in Materials and Methods. In all cell lines, GFP-MHC I heavy chain localized in the GA, which had the normal 'ring-like' appearance in PalF and no-E5 cells, whereas it was swollen and distorted in 4-E5 cells (Figure 3.3 A). To investigate whether E5 and MHC I co-localize, the cell lines were co-transfected with GFP-MHC I heavy chain and a plasmid encoding a fusion protein of the fluorescent protein DsRed and BPV-4 E5 (RFP-E5 described in chapter 2, sections 2.1.10 and 2.1.11). Like wild type E5 (Pennie *et al.* 1993), RFP-E5 localized in the GA (Figure 3.3 B). In all cell lines, GFP-MHC I heavy chain co-localized with RFP-E5 in the GA (Figure 3.3 B).

Figure 3.3

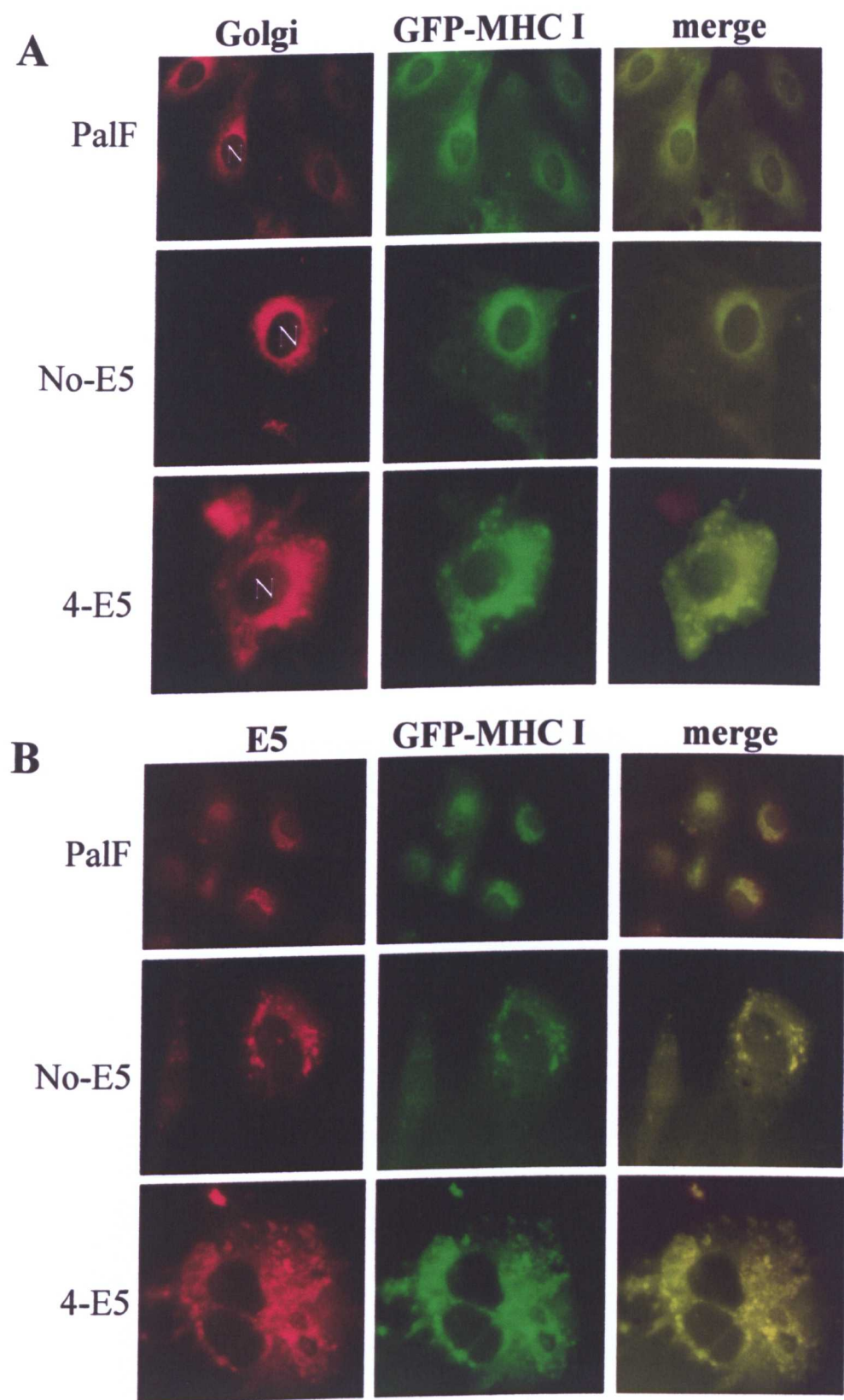


Figure 3.3 MHC I heavy chain and E5 co-localize in the GA

A) Cells stained with BODIPY-TR-Ceramide (red) to detect the GA and transfected with GFP-MHC I heavy chain (green). GFP-MHC I heavy chain localizes in the GA in all cell lines (experiment done by myself and figure published in Marchetti *et al.*, 2002).

B) Cells transfected with RFP-E5 (red) or GFP-MHC I heavy chain (green). MHC I heavy chain co-localizes with E5 in the GA in all cell lines. The images were collected with a LEICA DMLB fluorescence microscope equipped with a SenSys 1400 camera. Data sets were processed using the QUIPSTM Smart Capture imaging software (Vysis, UK). The merge between BODIPY-TR and GFP-MHC I or GFP-MHC I and RFP-E5 fluorescent signals was achieved using the merge option in the QUIPSTM imaging software (Vysis, UK). N= nucleus. Magnification 20x for PalF cells in A,B and 40x for no-E5 and 4-E5 cells in A,B (experiment done by myself and figure published in Marchetti *et al.* 2002).

3.3.1 Retention is specific

It was important to demonstrate that retention of MHC I in the GA was specific, and not due, for instance, to the nature of the fusion proteins used in the study. Therefore, the cells were transfected with a plasmid expressing the full length papillomavirus E2 protein (pCMV-E2 described in chapter 2, sections 2.1.10 and 2.1.11). The E2 protein was detected by indirect immunofluorescence with a mouse monoclonal antibody TVG261 directed against amino acids 2–17 in the amino terminus of HPV-16 E2. As expected (Ham *et al.* 1991), E2 localized in the nucleus in all cell lines, including 4-E5 transformed cells (Figure 3.3.1 A), showing that E5 did not retain a predominantly nuclear protein in the endomembrane compartment. Moreover, co-expression of E2 and GFP-MHC I heavy chain, or E2 and RFP-E5 did not lead to co-localization: E2 was nuclear in all cases, and MHC I heavy chain and E5 were localized in the GA (Figure 3.3.1 B).

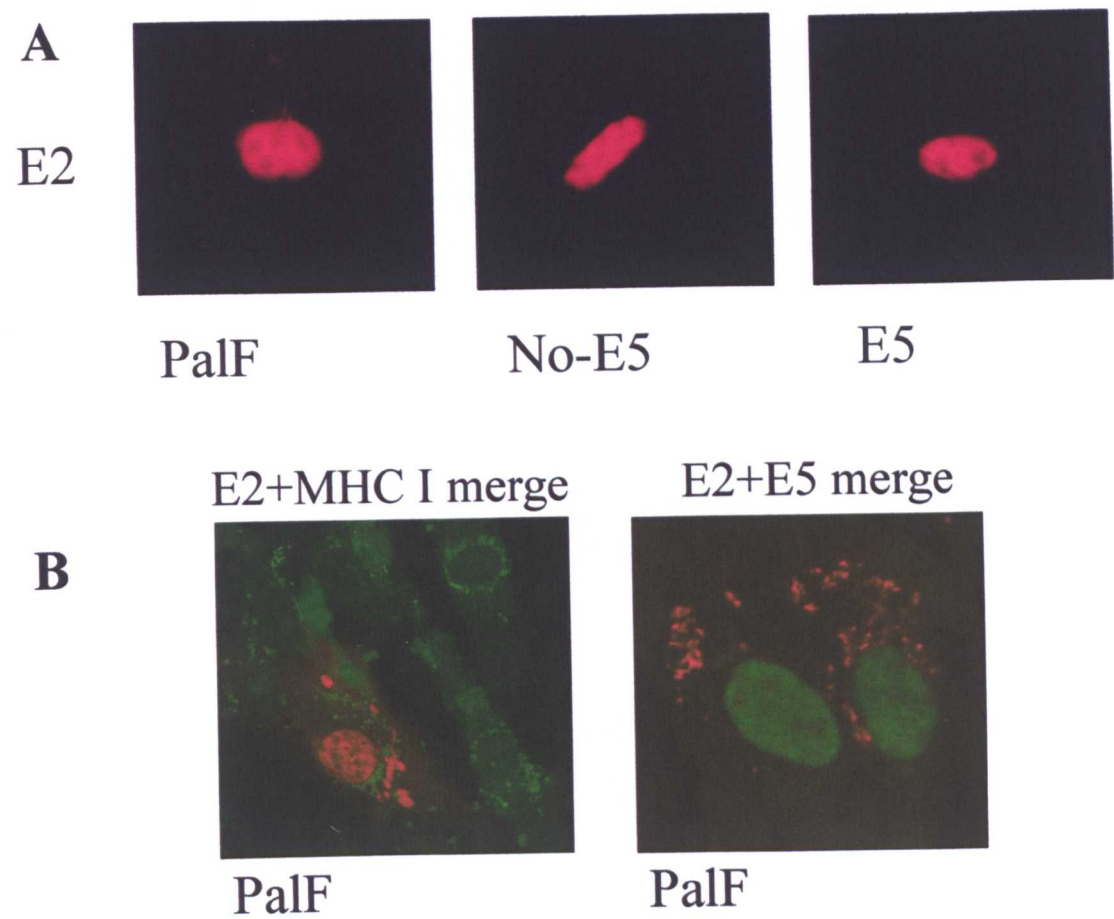


Figure 3.3.1 E2 localizes to the nucleus and does not co-localize with either MHC I heavy chain or E5

A) Cells were transfected with E2 only, or B) co-transfected with E2 and GFP-MHC I or E2 and RFP-E5. E2 was detected with mAb TVG216 and Texas Red-conjugated secondary antibody in (A) and in the left panel of (B), and with a FITC-conjugated secondary antibody in the right panel of (B). GFP-MHC I and RFP-E5 were visualized by direct immunofluorescence. The images were collected with a Zeiss LSM 150 confocal microscope utilising a x63 oil immersion objective lens, NA 1.4. Data sets were processed using the LSM 510 software. In B the figure that shows the merge E2+E5 was enlarged for ease of visualization (experiment done by myself and figures published in Marchetti *et al.* 2002).

3.4 Prevention of GA acidification by the ionophore monensin prevents transport of MHC I to cell surface

The monovalent ionophore monensin is widely used to investigate the function of the GA and vesicular transport (Bachert *et al.* 2001; Halaban *et al.* 2002; Schoonderwoert *et al.* 2002). Monensin disperses the proton gradient across the cell membranes and impedes the proper acidification of Golgi cisternae by displacing and inhibiting the H⁺-V-ATPase proton pump, leading to GA swelling and fragmentation and faulty protein transport from the medial to the trans-Golgi (Tartakoff 1983; Boss *et al.* 1984; Zhang *et al.* 1996; Chikuma *et al.* 2002). E5 complexes with 16kDa subunit c, a component of the V0 sector of the H⁺-V-ATPase (Goldstein *et al.* 1991; Conrad *et al.* 1993; Faccini *et al.* 1996; Ashrafi *et al.* 2002) and inhibits endomembrane acidification (Straight *et al.* 1995; Schapiro *et al.* 2000). In view of the similarity between the reported morphology of monensin-treated cells and E5 cells, and the functional similarity between monensin and E5, we treated PalF and no-E5 cells with monensin and analysed them for morphology, GA architecture and surface MHC I. Indeed, monensin-treated PalF cells or no-E5 cells showed extensive vacuolization and a grossly deformed GA (Figure 3.4 A, shown only for PalF cells). These results indicate that the vacuolization and GA malformation in E5 cells are likely due to the impeded acidification of the GA membranes brought about by the viral protein. Next we investigated whether treatment with monensin resulted in a down-regulation of surface MHC I in either PalF or no-E5 cells. Cells were treated with 25 μ M monensin for 30 min, 3 or 12 h, and analysed for surface or total MHC I by FACS analysis with mAb IL-A19 as described in materials and methods. Monensin treatment caused a fourfold decrease in surface MHC I after 30 min in PalF cells and after 3 h in no-E5 cells (Figure 3.4 C). The levels of surface MHC I returned to those seen in non-treated

cells after 12 h in both cell lines (data not shown) in agreement with the reported ability of cells to recover from the effects of monensin treatment (Zhang *et al.* 1993). We do not know why no-E5 cells down-regulated surface MHC I in response to monensin less rapidly than PalF cells (3 h vs 30 min); the delay may be due to the expression of the other oncoproteins present in no-E5 cells. The experiment was repeated three times and the ratio between the mean fluorescence for total and surface (T/S) MHC I was calculated. The T/S ratio confirmed that monensin treatment reduces expression of surface MHC I after 30 min of treatment, the T/S ratio in PalF cells was increased approximately four-fold, and in no-E5 cells the highest T/S ratio was obtained after 3 h of treatment with an increase of approximately of two-fold (Figure 3.4 C). The transport to the cell surface of another membrane glycoprotein, the transferrin receptor, was not affected in E5 cells (Figure 3.5 A), and likewise there were no differences in the levels of surface transferrin receptor between cells treated and not treated with monensin (Figure 3.5 B) (Ashrafi *et al.* 2002). Monensin treatment did not significantly affect the levels of total MHC I in PalF cells and slightly decreased it in no-E5 cells, as judged by immunoblotting (Figure 3.4 B), and therefore these data show that monensin inhibited the transport of MHC I to the cell surface but not its expression. These results indicate that, following inhibition of GA acidification by monensin, transport of MHC I to the cell surface is impeded, and support the notion that E5 prevents MHC I transport through inhibition of GA acidification.

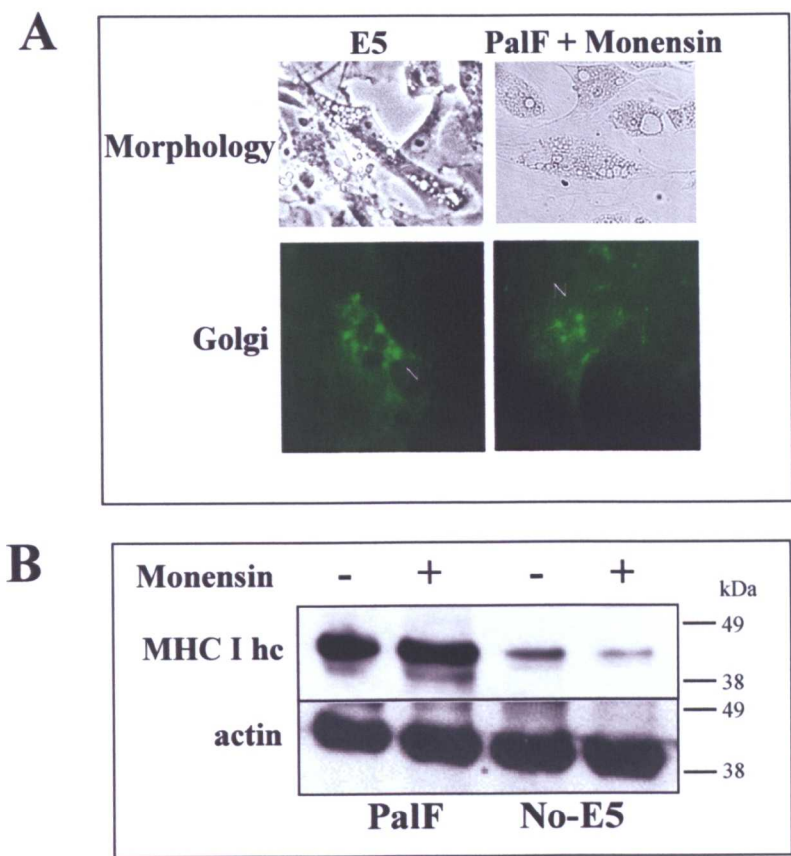


Figure 3.4 Monensin causes GA swelling and MHC I intracellular retention

A) Morphology and immunofluorescence of 4-E5 and PalF cells treated with monensin

The morphology of 4-E5 cells and PalF cells treated with 25 μ M monensin for 3 h was assessed using phase contrast microscopy (top panels), and immunofluorescence detection with mAb 4A3 to visualize the GA (bottom panels). Monensin-treated cells are highly vacuolated and present a swollen GA, similar to E5 cells, N=nucleus. Magnification 40x (experiment done by Dr. Hossein Ashrafi and figure published in Marchetti *et al.*, 2002)

B) Western blot analysis of monensin treated cells

PalF and no-E5 cells were treated with 25 μ M monensin for 3 h and 10 μ g of protein lysate were analysed by immunoblotting for MHC I expression. MHC I heavy chain (MHC I HC) was detected with mAb IL-A88 (top panel) and actin, as a loading control, with mAb AB-1 (bottom panel). Monensin does not affect the expression of MHC I heavy chain. The numbers on the right represent the molecular weights marker in kilodalton (kDa) (experiment done by myself and figure published in Marchetti *et al.* 2002)

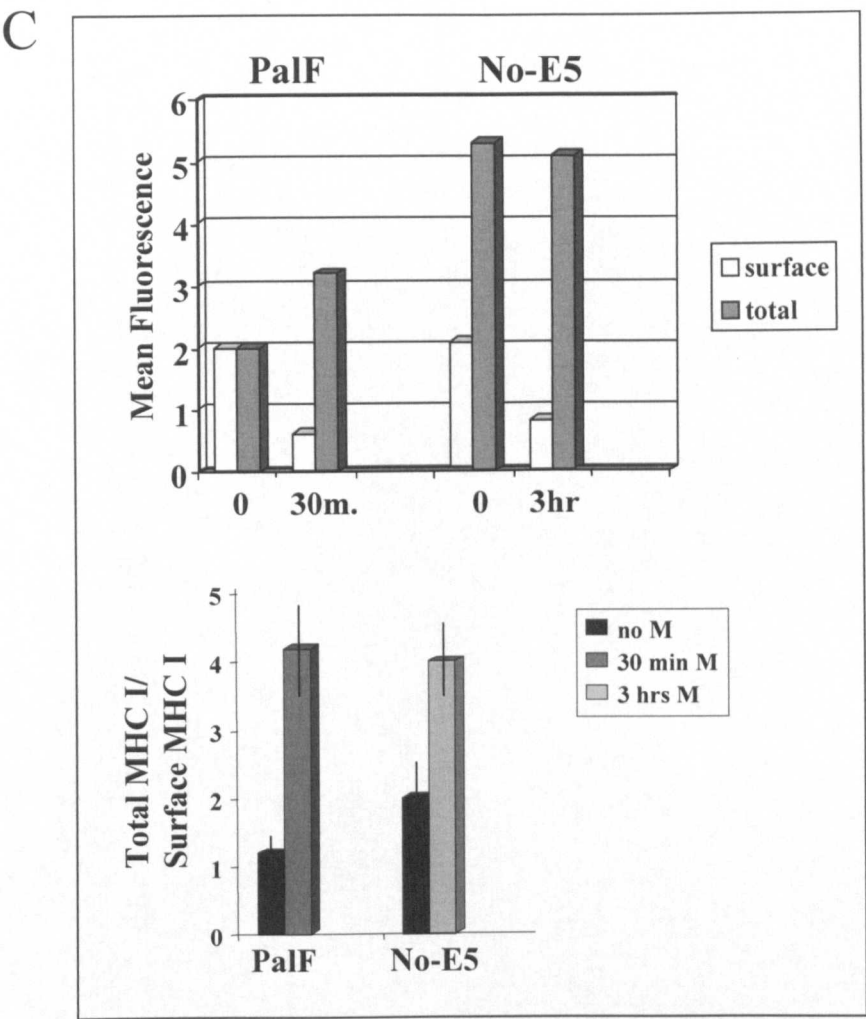


Figure 3.4 Monensin causes GA swelling and MHC I intracellular retention

C) FACS analysis of monensin treated cells

PalF and no-E5 cells were treated with 25 μ M monensin for 30 min or 3 h. Surface and total MHC I expression was examined by FACS analysis using mAb IL-A19. The mean fluorescence from one experiment is shown in the upper panel. Surface mean fluorescence decreased after 30 min treatment with monensin in PalF cells and after 3 h treatment in no-E5 cells. The ratio between total mean fluorescence and surface mean fluorescence (T/S) of PalF and no-E5 cells is shown in the bottom panel. The bars represent the average ratio from three experiments (\pm standard deviation). The T/S ratio increases approximately fourfold in PalF cells after 30 min of monensin treatment, and approximately twofold in no-E5 cells after 3 h of treatment, indicating a decrease in surface MHC I (experiment done by myself from data published in Marchetti *et al.* 2002).

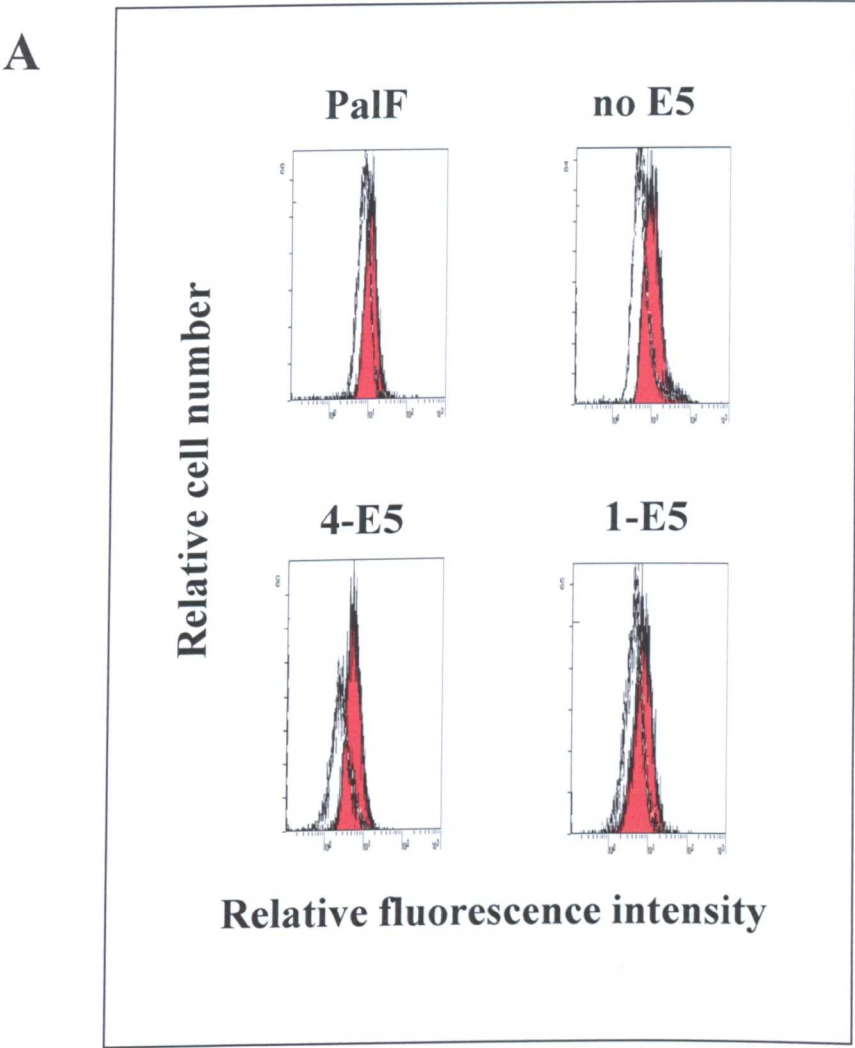


Figure 3.5 The transferrin receptor transport is neither affected by E5 or by monensin

A) FACS analysis of the transferrin receptor

Surface (solid histogram) transferrin receptor expression in parental and transformed PalF cells. Intact cells were stained with mAb IL-A165 and analysed by flow cytometry. The open histogram represent cells stained with FITC-conjugated secondary antibody only. The transport to the cell surface of the transferrin receptor is not affected in E5 cells (experiment done by Dr.Hossein Ashrafi and figure from data published in Ashrafi *et al.* 2002).

Figure 3.5

B

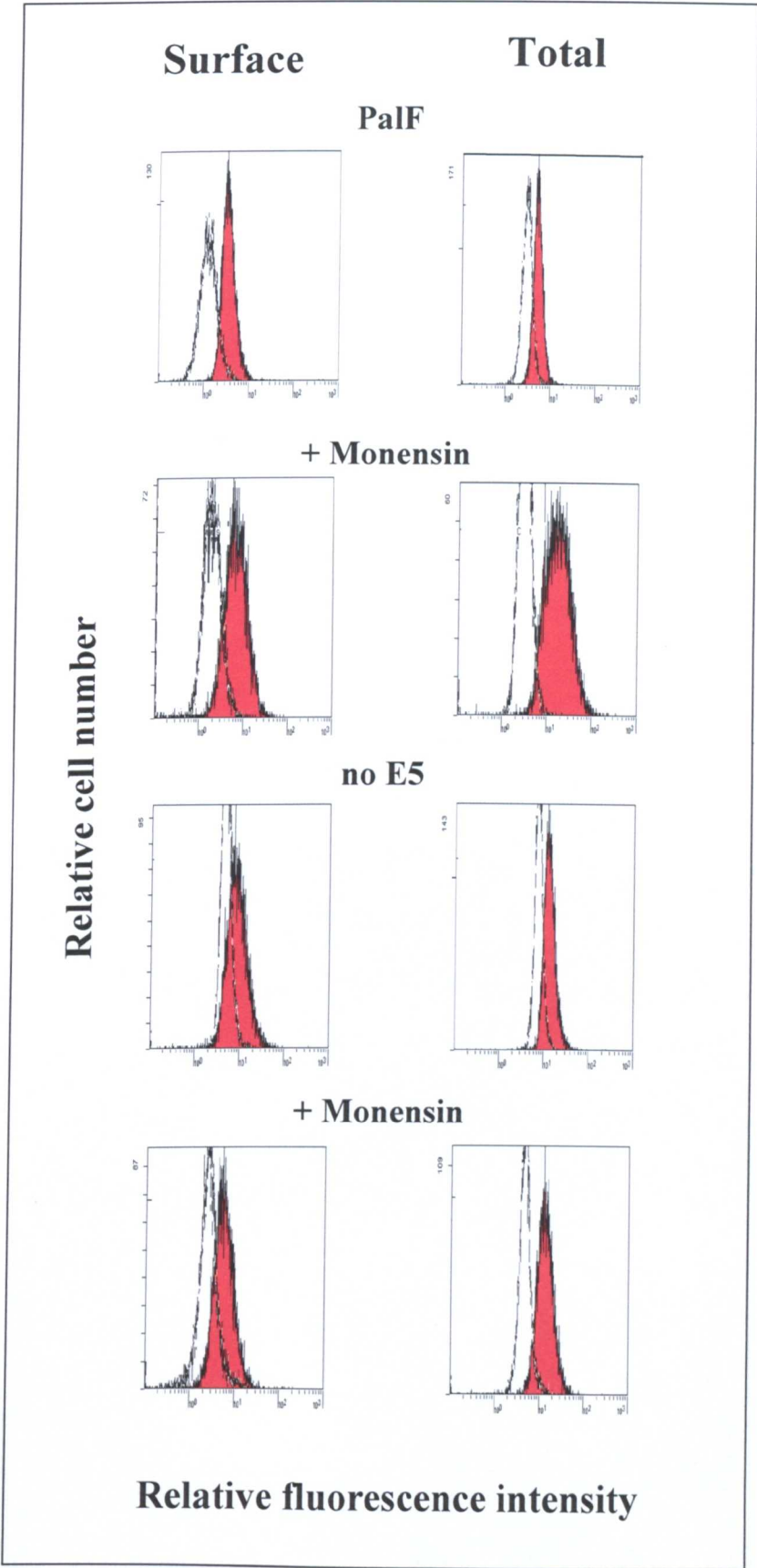


Figure 3.5 The transferrin receptor transport is neither affected by E5 or by monensin

B) FACS profile of transferrin receptor expression in monensin treated cells

PalF and no-E5 cells were untreated or treated with 25 μ M Monensin. Cells were stained with mAb IL-A165 and analysed by flow cytometry. The open histogram represent cells stained with FITC-conjugated secondary antibody only. The solid histogram represent either surface or total transferrin receptor expression.

There is no difference in the levels of surface transferrin receptor between cells treated or not treated with monensin (experiment done by Dr. Hossein Ashrafi, personal communication).

CHAPTER 4: Effect of the Bovine Papillomavirus Type 4 E5 protein on the transport of MHC class I molecule

4.1 Signal transduction by inteferons (IFNs)

Type I (α, β) and type II (γ) IFNs play a central role in the immune response. After receptor binding by α -IFN or β -IFN, specific tyrosine kinases, Tyk2 together with one or more additional tyrosine kinases, Janus-activated kinase (JAK)-1 and JAK-2, are phosphorylated. These activated tyrosine kinases activate the signal-transducing proteins (Stark *et al.* 1998) and induce the formation of a complex of protein subunits (interferon-stimulated gene factor [ISGF]-3 α) consisting of STAT-1 α or STAT- β and STAT-2. The phosphorylated ISGF-3 α complex is translocated to the nucleus and forms (with the addition of a fourth subunit p48 or IRF-9), a DNA-binding complex specific for the IFN-stimulated response element (ISRE). γ -IFN receptor activation results in a similar sequence of events, although the transcriptional regulatory complex consists of a homodimer of STAT-1 that binds to DNA elements termed gamma activated sites (GAS) (figure 4.1).

Both β -interferon and γ -interferon (β -IFN or γ -IFN) increase the transcription activity of the MHC I heavy chain gene by acting on the IFN-stimulated response element (ISRE), a conserved *cis*-acting regulatory element present in the promoter of MHC class I genes. The ISRE is a binding site for factors of the IFN-regulatory family (IRF) that form a group of secondary transcription factors that regulate gene transcription in a positive (IRF-1) or negative manner (IRF-2 and IFN consensus binding protein, ICSBP) or act as helpers of protein/DNA complex formation (p48

also known as IRF-9). (reviewed in (Agrawal and Kishore 2000; Taniguchi *et al.* 2001) (summarised in figure 4.1).

Figure 4.1

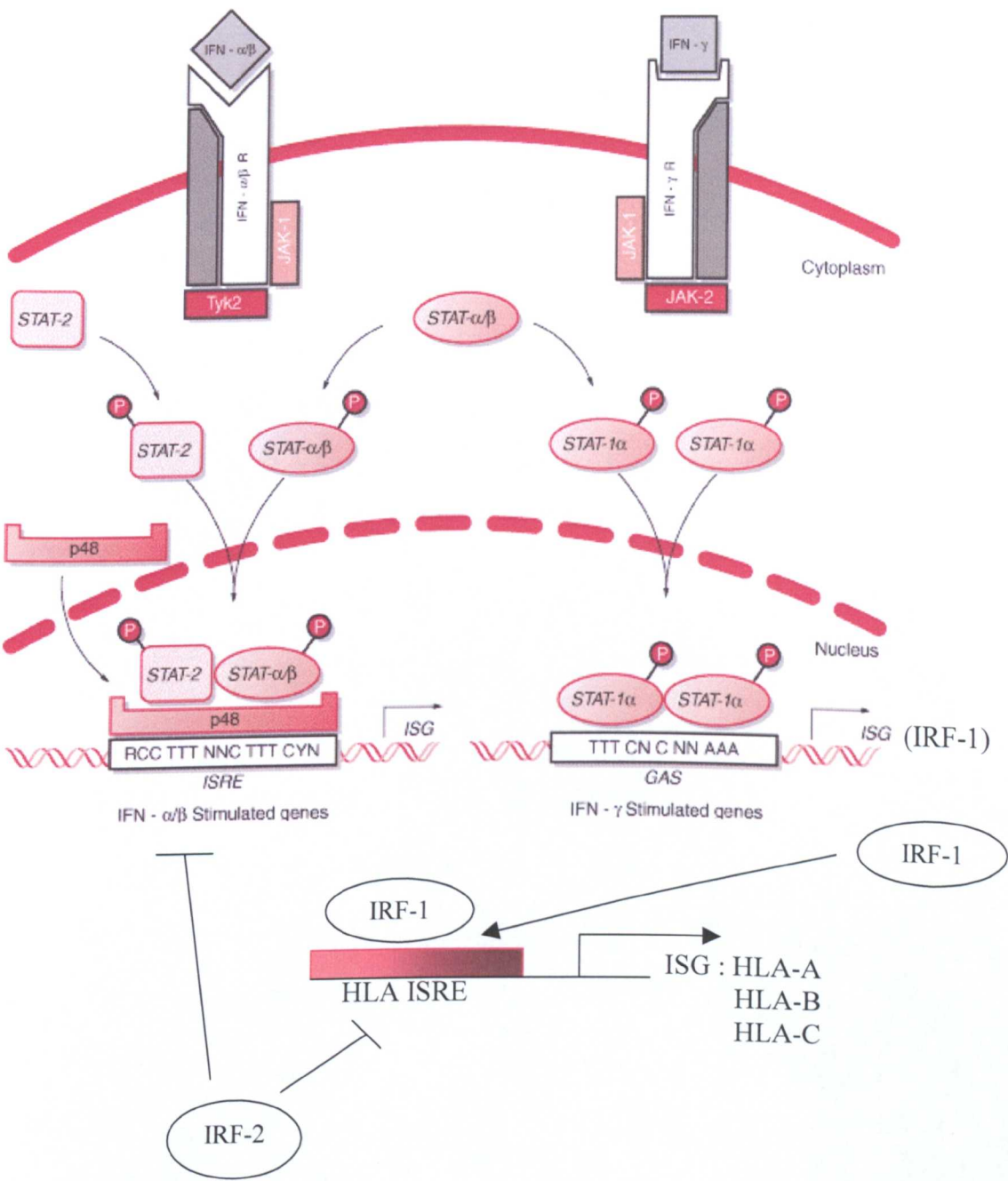


Figure 4.1 Pathway of signal transduction by IFNs

After binding to specific receptors, tyrosine kinases (JAK-1, JAK-2, Tyk2) are activated. These tyrosine kinases phosphorylate inactive transcription factors (STAT-1 α , STAT-1 β , STAT-2), which form a transcriptional factor, interferon-stimulated gene factor (ISGF)-3 α or STAT-1 α dimer. The phosphorylated ISGF-3 α complex is translocated to the nucleus and forms (with the addition of a fourth subunit p48), a DNA-binding complex specific for the IFN-stimulated response element (ISRE). The STAT-1 α dimer is also translocated to the nucleus and binds to DNA elements termed gamma activated sites (GAS). The interferon-stimulated response element (ISRE) and the gamma activation site (GAS) have shared nucleotides for all IFN-stimulated genes (ISG). IRF-1 gene is induced via GAS which is found within the IRF-1 promoter. IRF-1 then bind to ISRE, leading to the activation of IFN-inducible genes (such as HLA/MHC genes). N= nucleotide; Y= pyrimidine. (Picture from Cancer Medicine 2003 plus adaptation from data published by (Gobin *et al.* 1999; Taniguchi *et al.* 2001).

4.2 Treatment of E5 cells with interferon increases total but not surface MHC I

Previous work in this laboratory has shown that in E5 cells there is less MHC I heavy chain mRNA and protein (Ashrafi *et al.* 2002). To assess if the biosynthetic pathway of heavy chain is permanently inhibited by E5, PalF, no-E5 and E5 cells were treated with either β - or γ -IFN or 2D6, a control supernatant from cells not expressing IFN. Treatment with either IFN, but not with 2D6, increased production of the MHC I heavy chain in all cell lines as assessed by quantitative RT-PCR and immunoblotting (Figure 4.2 A,B) and noticeably the treatment does not increase expression of 4-E5 (Figure 4.2 C). This shows that E5 cells are responsive to IFN and the biosynthetic

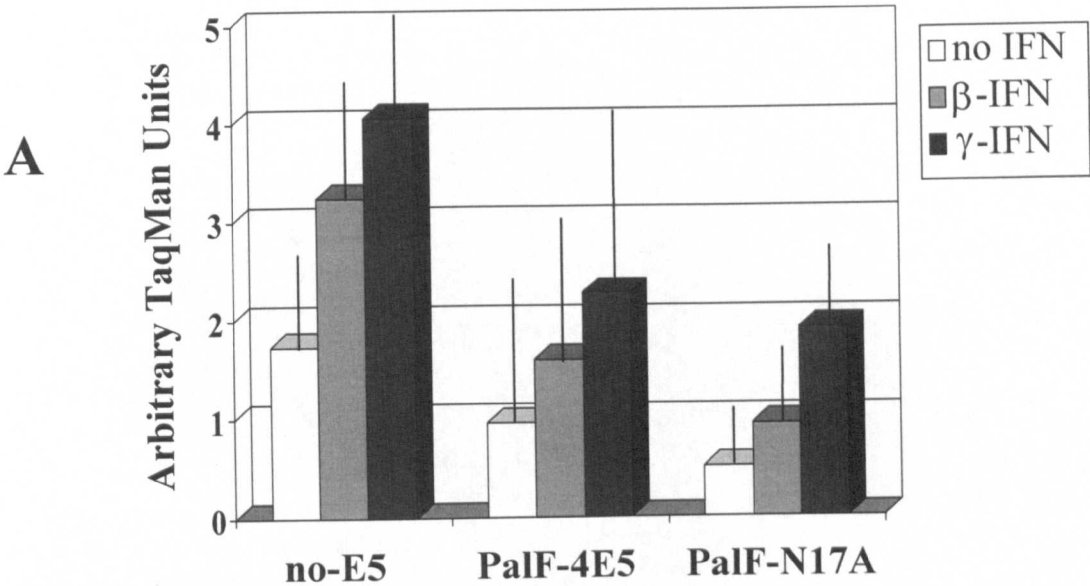
pathway of MHC I heavy chain is not irreversibly inhibited by E5 but the production of MHC I heavy chain is still lower than in no E5 cells.

To investigate whether the reduction in HC RNA was due to transcriptional repression or to degradation of the RNA, pBoLa-Luc, containing the reporter gene for luciferase under the transcriptional control of the promoter/enhancer of a bovine MHC class I HC gene, was transiently introduced into PalF cells along with pZip4-E5 (O'Brien *et al.* 1999) , a plasmid expressing BPV-4 E5. Expression of luciferase was less than half the level than when pBoLa-Luc was cotransfected with an empty pZip vector (Figure 4.2 D, white bars). This result indicates that the reduction in HC RNA in PalF 4-E5 cells is due to the E5-induced inhibition of the transcriptional promoter of the HC gene. Additionally, γ -IFN-treatment stimulated the expression of luciferase from pBoLa-Luc by approximately 2-fold both in the presence or absence of 4-E5 (Figure 4.2 D, grey bars). Even with γ -IFN-treatment, in cells expressing 4-E5 luciferase expression did not achieve the levels observed in cells that did not express E5.

These results show that 4-E5 inhibits transcription of cattle class I HC genes, and that IFN treatment rescues transcription, but without completely overcoming 4-E5-induced repression.

The IFN-treated cells were also analysed by FACS to ascertain whether the increased production of heavy chain led to a corresponding increase in surface MHC I. MHC I surface expression increased only in PalF and no-E5 cells but not in E5 cells, or in cells expressing the 4-E5 N17A mutant in which a substitution of the asparagine in position 17 to alanine resulted in hypertransforming characteristics (described in detail in section 5.2) (Figure 4.2 E), despite the increase in total MHC I. These results show that E5 inhibits the transport of MHC I from the endomembrane compartment to the cell surface.

Figure 4.2



B

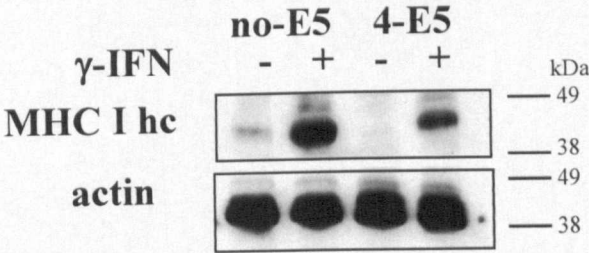
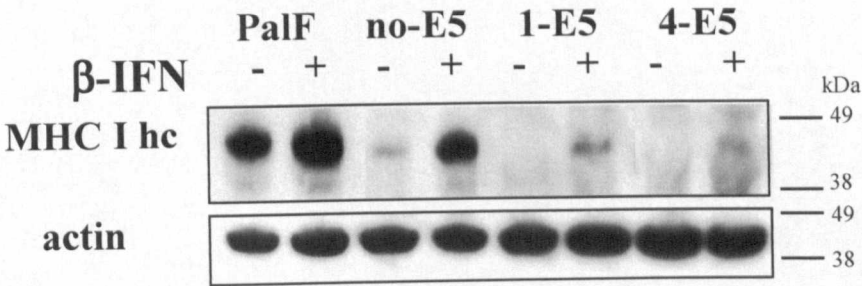


Figure 4.2 Treatment of E5 cells with interferon increases total but not surface MHC I**A) IFNs treatment increase MHC I heavy chain transcription**

Quantitative RT-PCR for MHC class I HC RNA was performed on RNA from no-E5 cells, PalF-4E5 cells and PalF cells expressing the hypertransforming 4-E5 mutant N17A (PalF-N17A), before (white bars) or after treatment with 500U/ml β -IFN (grey bars) or 250U/ml γ -IFN (black bars). The extent of amplification was normalised to that of the actin RNA and is plotted as arbitrary units. The panel shows the averages with standard deviations of at least three experiments. Both β -IFN and γ -IFN increased transcription of HC RNA in control and E5 cells. (experiment done by Dr. Rubina Ullah a former worker in this laboratory from data published in Marchetti *et al.*, 2006).

B) IFNs treatment increase MHC I heavy chain expression in all cells.

Cells were treated for 48h with 500 U/ml of either β -IFN or γ -IFN (lanes marked +), or with 500 U/ml of the control supernatant 2DG (lanes marked -) and 10 μ g of protein lysates were analysed by immunoblotting. MHC I heavy chain (MHC I HC) was detected with mAb IL-A88 (top panel) and actin, as loading control, with mAb AB-1 (bottom panel). The numbers on the right represent the molecular weights marker in kilodalton (kDa) (experiment done by Dr. Hossein Ashrafi from data published in Marchetti *et al.* 2002).

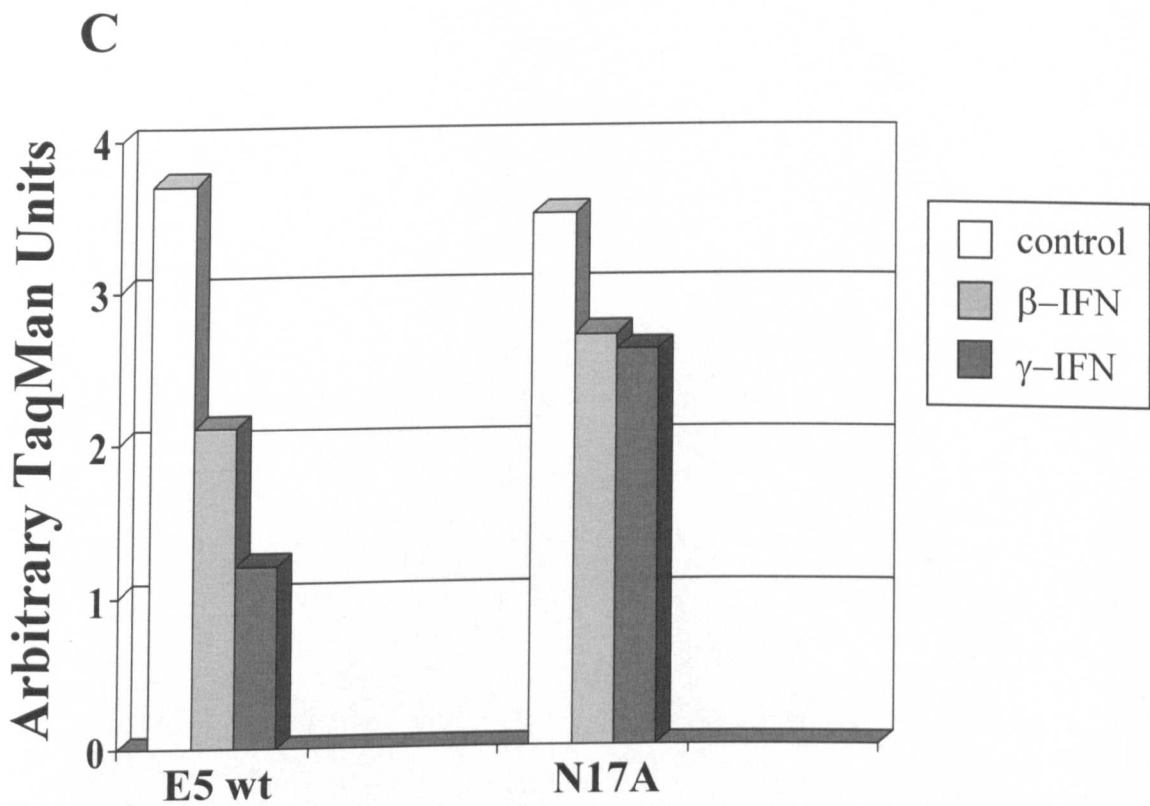


Figure 4.2 Treatment of E5 cells with interferon increases total but not surface MHC I

C) IFN treatment does not increase E5 mRNA

Quantitative RT-PCR for E5 RNA was performed on RNA from PalF 4-E5 cells and PalF-N17A cells before (white bars) or after treatment with 250U/ml β -IFN (grey bars) or 250U/ml γ -IFN (black bars). The extent of amplification was normalised to that of the actin RNA and is plotted as arbitrary units. The panel shows the averages of one experiment done in triplicate. (experiment done by Dr. Rubina Ullah a former worker in this laboratory).

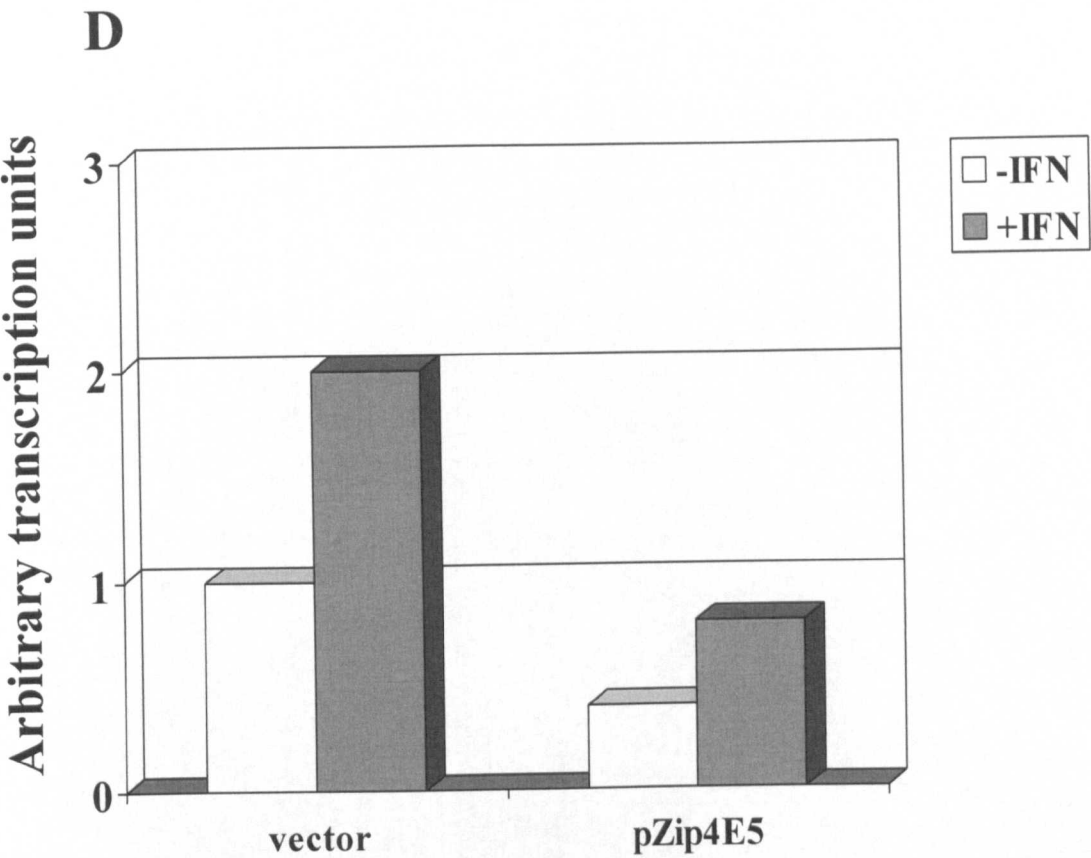


Figure 4.2 Treatment of E5 cells with interferon increases total but not surface MHC I

D) BPV-4 E5 inhibits transcription from the BoLa promoter

BoLa promoter transcriptional activity in PalF parental cells transiently cotransfected with pBoLa-Luc and pZipneo empty vector, or with pBoLa-Luc and pZip-4E5 (O'Brien et al., 1999), either without (white bars) or with 250 U/ml γ -IFN treatment (grey bars). Luciferase activity is plotted as arbitrary transcription units, with the activity of pBoLa in PalF parental cells taken as 1. γ -IFN increases the transcriptional activity of pBoLa also in presence of E5. (experiment done by Wendy Crawford a former student in this laboratory and picture from data published in Marchetti et al., 2006).

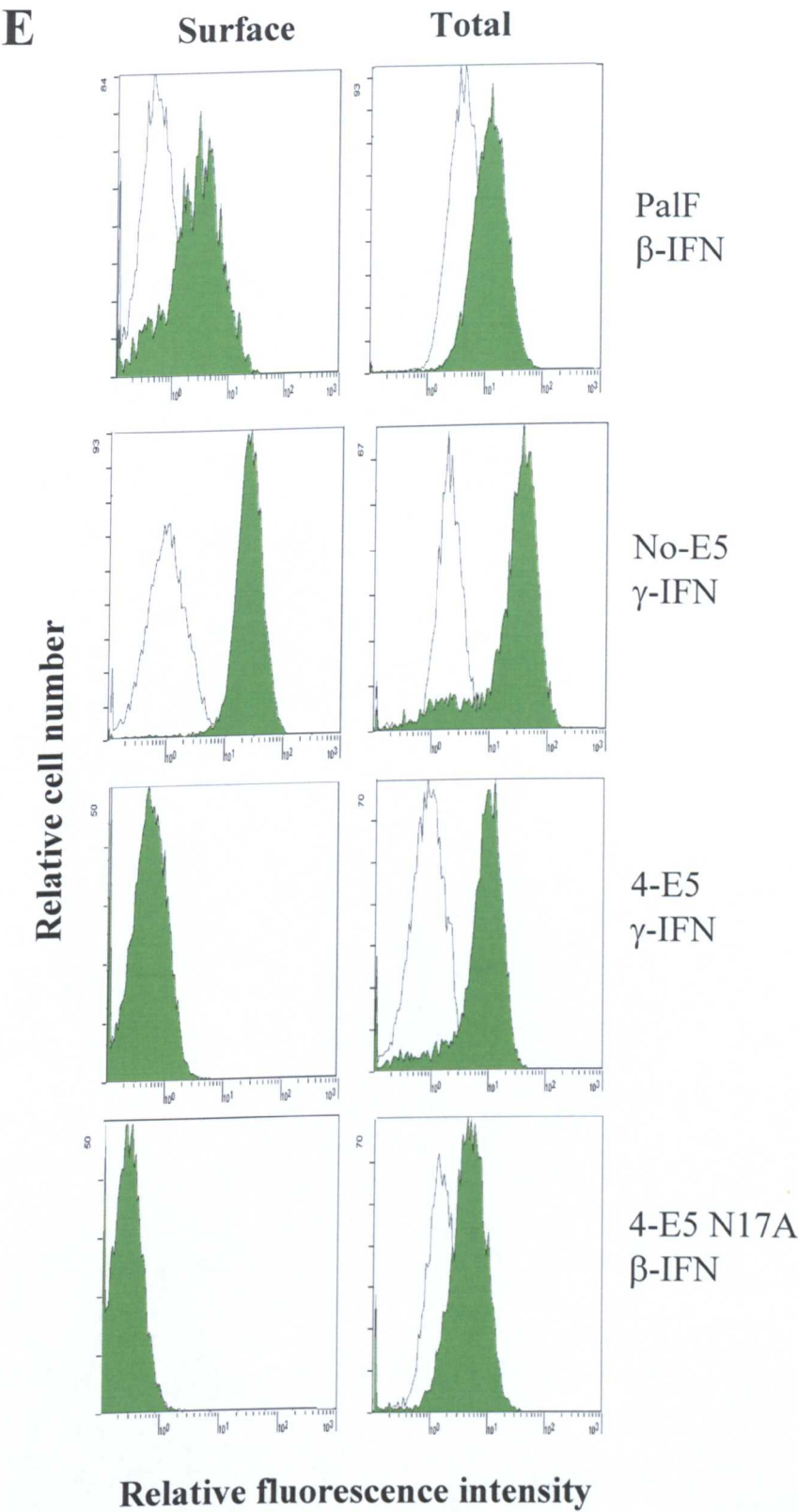


Figure 4.2 Treatment of E5 cells with interferon increases total but not surface MHC I

E) FACS profile of MHC I surface and total expression. MHC I is not transported to the cell surface in IFN-treated E5 cells. Cells treated with β -IFN (PalF, 4-E5 N17A) or γ -IFN (no-E5, 4-E5) (solid histograms), or the control supernatant 2DG (open histograms) were analysed for expression of total and surface MHC I by FACS analysis with mAb IL-A19. IFN treatment increases both total and surface MHC I in PalF and no-E5 cells, but only total MHC I in E5 cells or cells expressing the hypertransforming mutant N17A. (experiment done by Dr. Hossein Ashrafi from data published in Marchetti *et al.*, 2002).

4.3 Lysosome and proteasome inhibitors increase the stability of the heavy chain in E5-expressing cells

MHC class I stability is regulated by proteasomes and lysosomes, which degrade mislocated or mis-folded MHC class I molecules (Bartee *et al.*, 2004; Hewitt *et al.*, 2002; Hughes *et al.*, 1997). The levels of HC protein are extremely low in PalF E5 cells, but increase when transcription of the HC gene is stimulated by IFN treatment, without however reaching the levels of HC in IFN-treated control cells (Figure 4.3). To investigate further the cause of this reduction in HC protein, the no-E5 and 4-E5 cell lines were treated with inhibitors of lysosomes (bafilomycin or ammonium chloride) or of proteasomes (MG132 or ALLN).

Ammonium chloride is a known lysosomotropic agent which enter the lysosome and neutralize the H^+ ions (Poole *et al.* 1977); Bafilomycin is a macrolide antibiotic that acts as a specific inhibitor of vacuolar-type H^+ -ATPase that play a pivotal role in acidification and protein degradation in the lysosomes *in vivo* (Yoshimori *et al.* 1991);

MG132 is a potent cell-permeable proteasome inhibitor that reduces the degradation of ubiquitin-conjugated proteins by the 26S complex in mammalian cells (Rock *et al.* 1994) and ALLN (also known as MG101) is a potent cysteine protease and proteasome inhibitor (Vinitsky *et al.* 1992).

Levels of MHC class I were assessed by immunoblotting. Treatment with inhibitors increased the amounts of HC in no-E5 cells but only marginally in 4-E5 cells (Figure 4.3, lanes with no IFN), probably because of the preceding block of HC gene transcription. Given that the transcriptional block could be relieved by IFN, cells were treated with both IFN and inhibitors. After the combined treatment, the level of HC protein in 4-E5 cells was comparable with that of no-E5 cells (Figure 4.3). It is reasonable to conclude that in E5 cells, as in control cells, the degradation of the HC protein via the lysosomes and proteasomes can be abrogated by treatment with inhibitors.

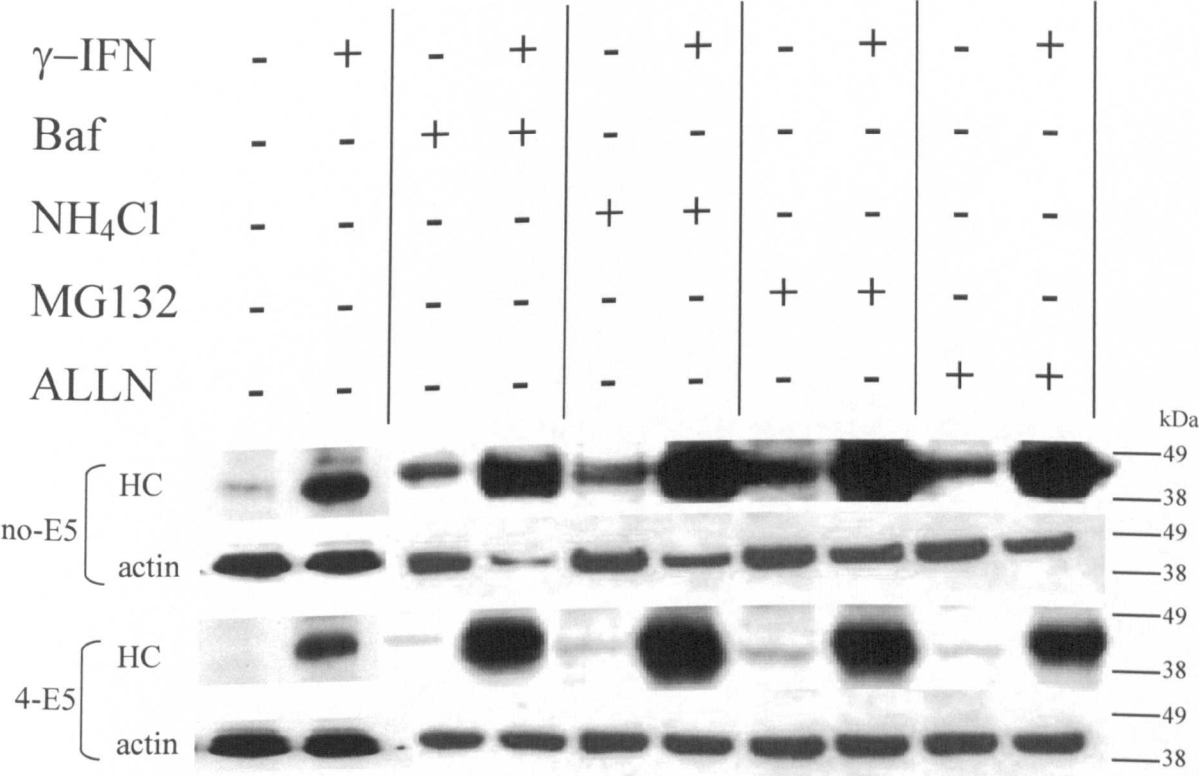


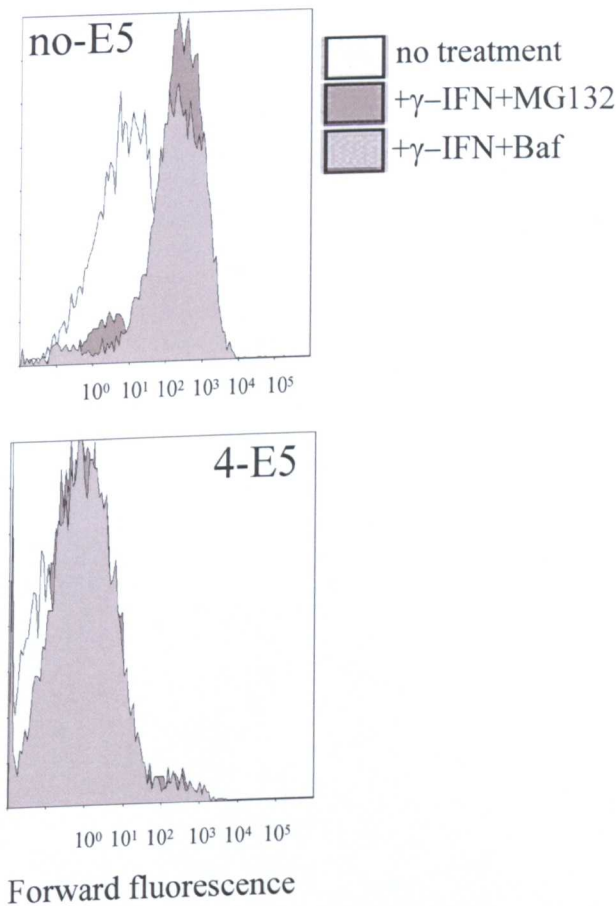
Figure 4.3 Proteasome and lysosome inhibitors prevent degradation of MHC class I HC in control and E5 cells.

Cells were left untreated or treated with either 250U/ml γ -IFN for 48 h, the lysosome inhibitors Bafilomycin A1 (1 μ M) or NH₄Cl (40mM) for 24 h, or the proteasome inhibitors MG-132 (5 μ M) or ALLN (100 μ M) for 8 h. For combined treatment with γ -IFN and inhibitors, lysosome inhibitors were added for the last 24 h of γ -IFN treatment, and proteasome inhibitors for the last 8 h. Protein lysates were probed with mAb IL-A88 against HC, or with mAb AB-1 against actin. Combined γ -IFN and inhibitors treatment increases levels of MHC class I HC in both control and E5 cells. The experiment shown is representative of at least three experiments giving essentially the same results. The numbers on the right represent the molecular weights marker in kDa (experiment done by myself from data published in Marchetti *et al.*, 2006).

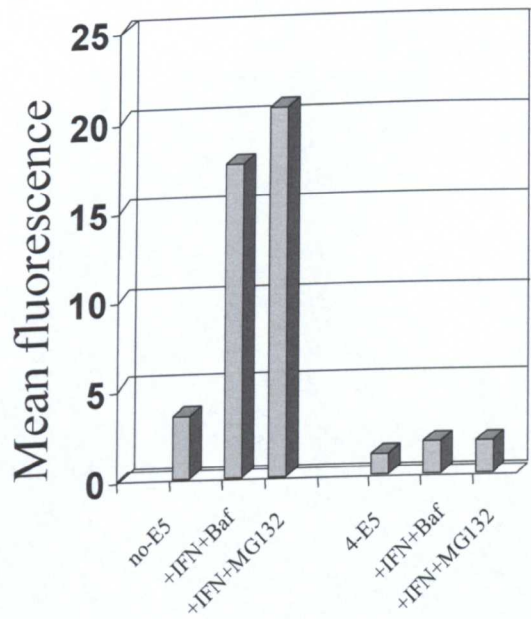
4.4 Transport of MHC class I complex to the cell surface is irreversibly inhibited by BPV-4 E5

To see whether the increase in MHC class I HC induced by treatment with IFN and lysosome or proteasome inhibitors resulted in increased levels of MHC class I complex on the cell surface, flow cytometry analyses of the treated cells were performed. Treatment with IFN+bafilomycin or IFN+MG132 increased the amount of surface MHC class I in no-E5 cells by 5-6 fold, but not in PalF 4-E5 cells, where the increase was so negligible that the shift in forward fluorescence could not even be detected (Figure 4.4 A,B). These results show conclusively that the 4-E5 protein prevents the traffic of MHC class I complex to the cell surface in an irreversible manner.

A



B



**Figure 4.4 MHC class I transport to the cell surface is irreversibly inhibited
by BPV-4 E5**

Cells were treated with γ -IFN + MG-132 or γ -IFN + Bafilomycin A1, as in Figure 4.3 and expression of surface MHC class I was analysed by flow cytometry with mAb IL-A19.

A) FACS profiles of control no-E5 and 4-E5 cells with no treatment (white), γ -IFN + MG-132 (dark grey) and γ -IFN + Bafilomycin A1 (light grey).

B) The extent of surface MHC class I expression is plotted as Mean Forward Fluorescence. In control cells, combined γ -IFN and inhibitors treatment increases surface MHC class I, whereas in 4-E5 cells surface MHC class I does not increase. The experiment shown is representative of three experiments giving essentially identical results (experiment done by myself from data published in Marchetti *et al.*, 2006).

CHAPTER 5: Analysis of the effects of BPV-4 E5 wild type protein and mutants on the inhibition of MHC I cell surface transport: physical interaction between BPV-4 E5 and MHC class I heavy chain

5.1 *In vitro* interaction between BPV-4 E5 and classical MHC class I heavy chain

The retention of MHC class I in the Golgi apparatus is due at least in part to the alkalinisation of the organelle (Marchetti *et al.* 2002), attributed to the binding of E5 to the 16k subunit c of the vacuolar H⁺ ATPase (Goldstein *et al.* 1991). However, the almost complete co-localisation of 4-E5 and the residual MHC class I in the Golgi apparatus (Marchetti *et al.* 2002) would suggest additional mechanisms. To further investigate this hypothesis, *in vitro* co-immunoprecipitation experiments were performed. First, 4-E5 was cloned in a suitable plasmid for transcription translation assay (pcDNA3.1(-)), N*01301 cDNA, encoding the bovine HC N*01301 (formerly known as HD6) was already cloned in a suitable plasmid (pcDNA3) under the control of a T7 promoter (Ellis *et al.* 1996; Gaddum *et al.* 1996; Ellis *et al.*, 1999) (details of constructs in chapter 2, sections 2.1.10 and 2.1.11). The efficiency of translation was assessed by *in vitro* transcription/translation assay (as described in Materials and Methods section 2.2.4.7) (fig. 5.1 A).

4-E5 and the bovine N*01301 HC were separately transcribed/translated *in vitro* in the presence of ³⁵S-methionine (Faccini *et al.* 1996) and then kept separately or mixed together. The proteins were precipitated either with Ab 274 against the C-terminus of E5 (Anderson *et al.* 1997; Araibi *et al.* 2004) or mAb IL-A88 against bovine HC. There was no precipitate in the absence of antibody (Figure 5.1 D, lanes 3,5,7); the

anti-E5 antibody precipitated 4-E5 but not N*01301 HC (Figure 5.1 B, lanes 3,2), and mAb IL-A88 precipitated HC but not 4-E5 (Figure 5.1 D lanes 4,6). However, when the two proteins were mixed together, they co-precipitated with either antibody (Figure 5.1 B, lane 4 and 5.1 C, lane 3), indicating that 4-E5 and HC interact, at least *in vitro*. This interaction is not an artefact as 4-E5 is precipitated by an antibody against HC even when N*01301 HC is not labelled (Figure 5.1 C, lane 5).

To see whether the interaction between 4-E5 and HC was specific for a particular MHC haplotype, the experiment was repeated with a different HC, N*00201 (formerly known as JSP.1) (Pichowski *et al.* 1996). Also in this case, 4-E5 and N*00201 HC co-precipitated when incubated with mAb IL-A88 (Figure 5.1 D, lane 8), showing that interaction is not restricted to certain MHC alleles.

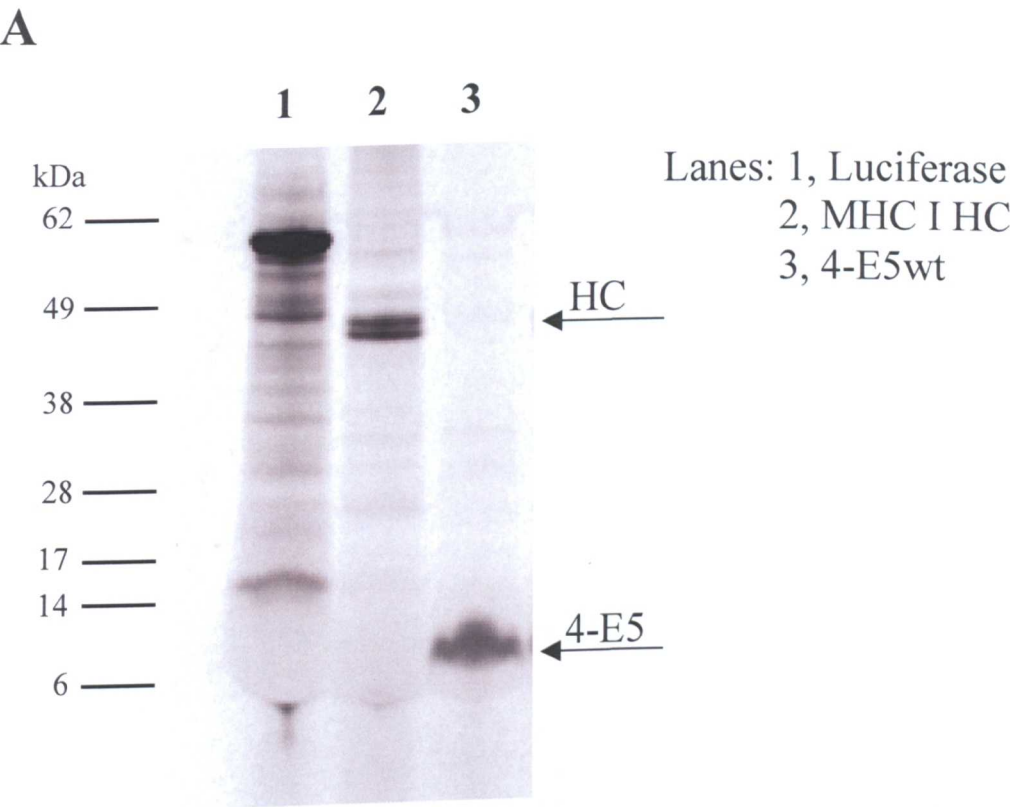


Figure 5.1 BPV-4 E5 and MHC class I HC interact *in vitro*

A) Transcription/translation assay

1µg of each plasmid encoding for the 4-E5 protein, the bovine N*01301 HC and the luciferase control DNA was *in vitro* transcribed/translated in presence of ³⁵S-methionine. The produced proteins were run on a 4-12% NuPAGE gel and the dried gel exposed on a screen and analysed on a Storm 840 apparatus. Luciferase was run as an internal control in the transcription/translation reaction. The numbers on the left represent the molecular weights marker in kilodalton (experiment done by myself).

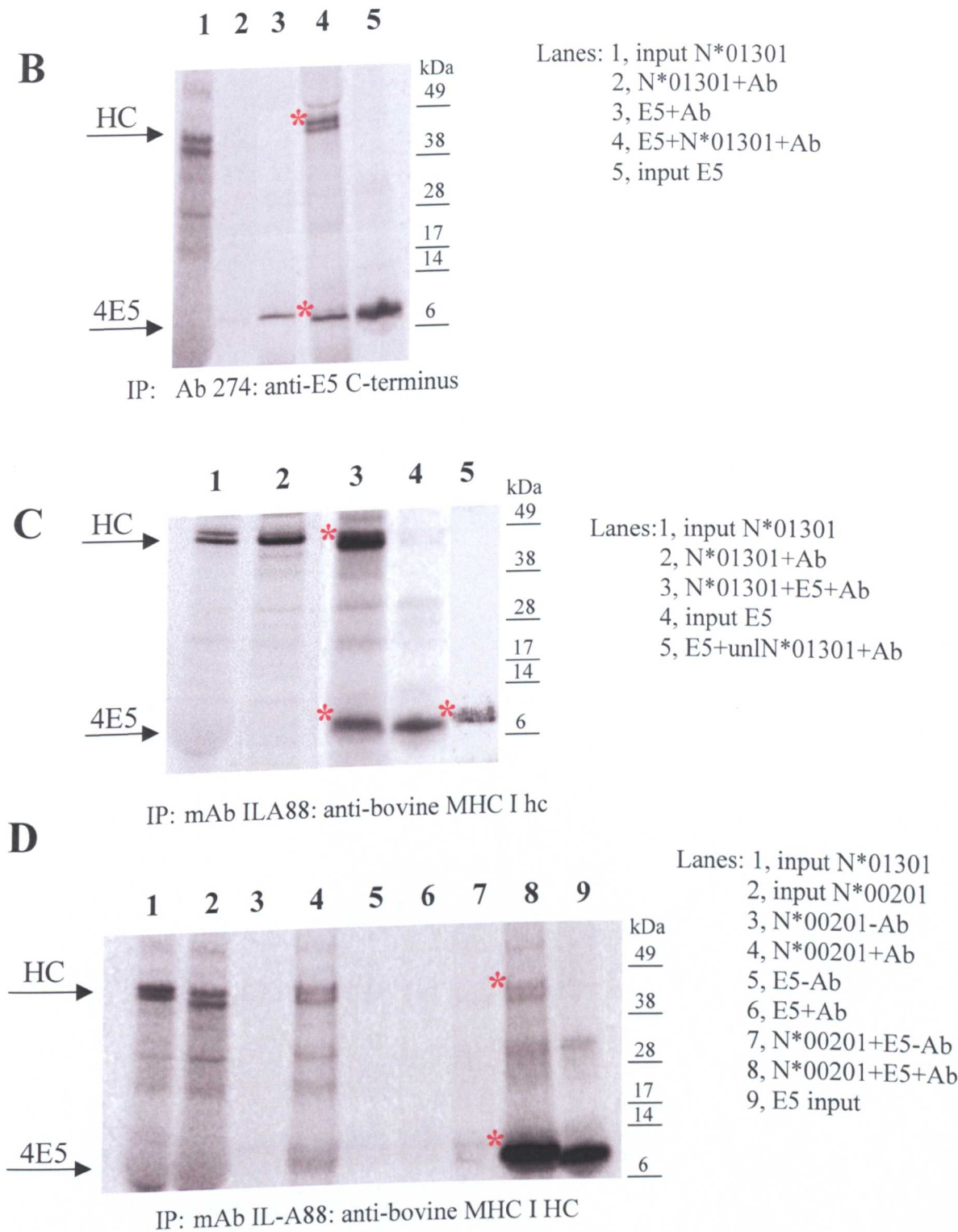


Figure 5.1 BPV4-E5 and MHC class I HC interact *in vitro****In vitro* co-immunoprecipitation.**

For ease of description, 4-E5 wt will be referred to as E5; the red stars indicate the interaction between E5 and either N*01301(B and C) or N*00201(D). The numbers on the right represent the molecular weights marker in kilodalton.

B) ³⁵S-labelled *in vitro* transcribed/translated E5, and N*01301 HC were immunoprecipitated with Ab 274, against the C-terminus of E5; the precipitated proteins were run on a NuPAGE gel and the gel dried and exposed on a screen then analysed on a Storm 840 apparatus. E5 and N*01301 HC interact physically.

C) ³⁵S-labelled *in vitro* transcribed/translated E5 and N*01301 HC were immunoprecipitated with mAb IL-A88 against bovine MHC class I HC; the precipitated proteins were processed as in A. E5 is precipitated by mAb IL-A88 even when N*01301 is unlabelled (unlN*01301) (red star, lane 5). N*01301 HC and E5 interact physically. Note that lanes 1-4 and 5 were run in separate gels and aligned with the other lanes for ease of comparison.

D) ³⁵S-labelled *in vitro* transcribed/translated E5 and N*00201 HC were immunoprecipitated with mAb IL-A88 against bovine MHC class I HC and processed as in A. N*00201 HC and E5 interact physically. (experiments done by myself from data published in Marchetti *et al.*, 2006).

5.2 BPV-4 E5 mutants that do not transform cells do not downregulate MHC I

In a study of the cell transforming properties of E5 (O'Brien *et al.* 1999), this laboratory established that mutation of the asparagine residue 17 of BPV-4 E5 to tyrosine (N17Y) lead to loss of cell transformation and so did deletion of the C-

terminus (E5T) of both BPV-4 and BPV-1 E5. On the contrary, mutation of N17 of BPV-4 E5 to alanine (N17A) resulted in a hyper-transforming mutant. Further studies established that the ability to down-regulate MHC class I expression co-segregated with the ability to transform cells (Ashrafi *et al.* 2002; O'Brien and Campo 2003). 4-E5 mutants that did not transform did not down-regulate MHC class I as assessed by flow cytometry (Figure 5.2 B) and both the total and cell surface levels of MHC class I were similar to those of control cells. In contrast the hyper-transforming mutant down-regulated surface MHC class I to the same extent as wild type 4-E5 (Figure 5.2 B). Figure 5.2 A is a schematic representation of 4-E5 wild type protein and its mutants. In table 5.3 the known characteristics of cells expressing 4-E5 or its mutants are summarised (Faccini *et al.* 1996; Ashrafi *et al.* 2000; Ashrafi *et al.* 2002).

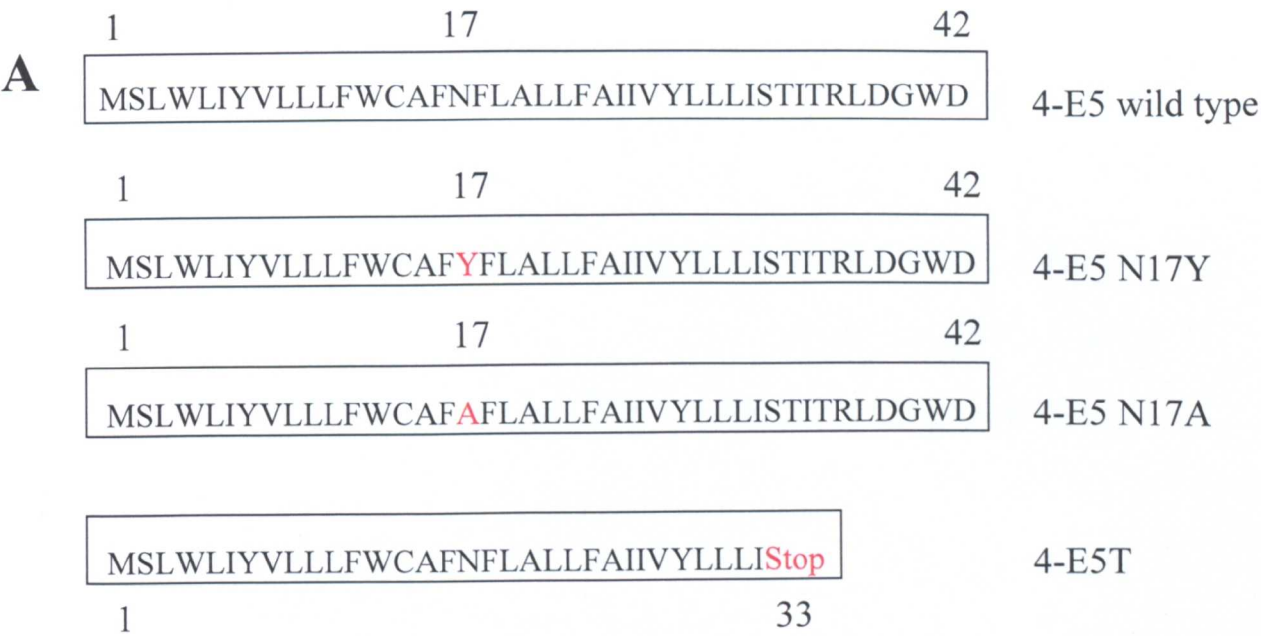


Figure 5.2 E5 mutants that do not transform do not down-regulates MHC class I

A) Schematic representation of BPV-4 E5 wild type protein and its mutants

The mutated amino acids are in red.

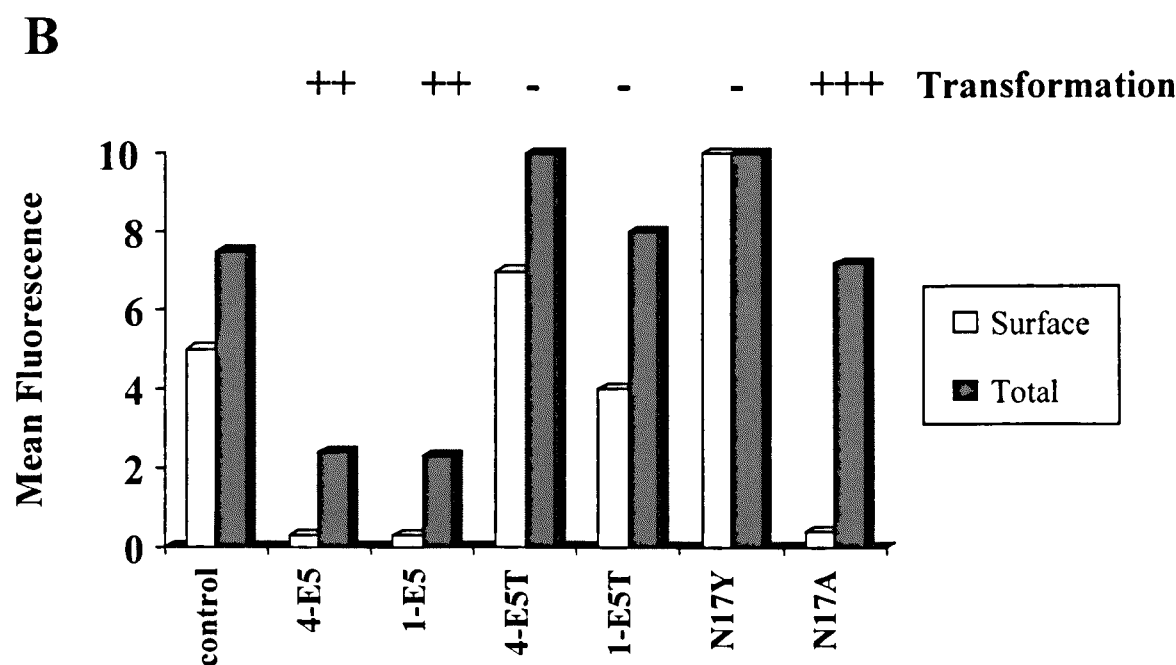


Figure 5.2 E5 mutants that do not transform do not down-regulates MHC class I

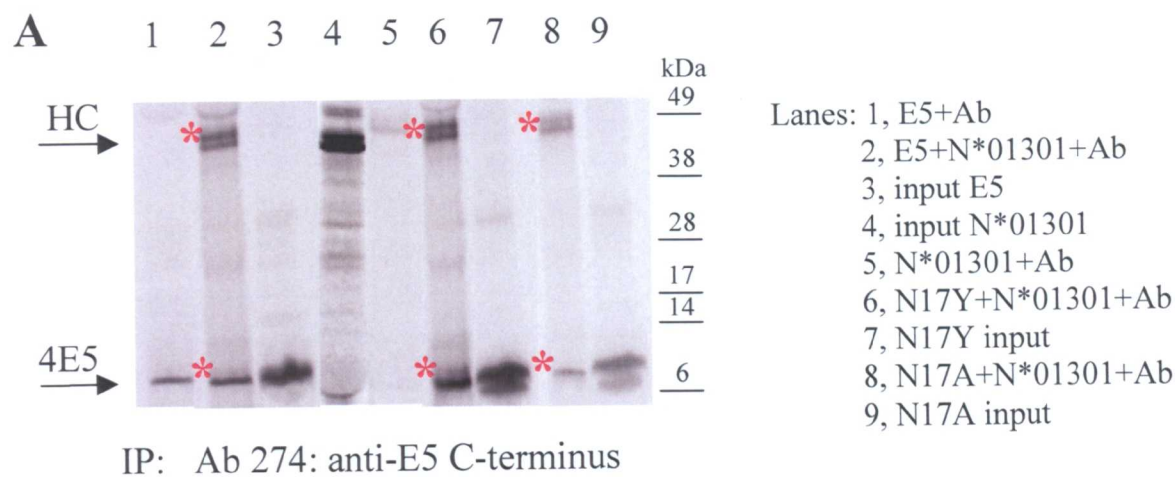
B) Representative mean fluorescence of surface or total MHC I

Surface and total MHC I in transformed PalF cells expressing mutant forms of 4-E5 (N17Y, N17A, 4-E5T) and of 1-E5 (1-E5T). The plus and minus signs above the histogram refer to the degree of cell transformation (experiments done by Dr. Hossein Ashrafi from data published in Ashrafi *et al.* 2002).

5.3 Mutants and classical MHC class I heavy chain: BPV-4 E5 inhibits MHC class I transport and binds to MHC heavy chain via its C-terminus.

To investigate whether the 4-E5 mutants were capable of interacting with HC, co-immunoprecipitation experiments were performed as described above. Both N17Y and N17A were co-precipitated with HC by Ab 274 (Figure 5.3 A, lanes 6,8) and mAb IL-A88 (Figure 5.3 B, lanes 5,7), while 4-E5T was not co-precipitated with N*01301 HC by mAb IL-A88 (Figure 5.3 B, lane 9). 4-E5T lacks the C-terminal domain and therefore could not be precipitated by Ab 274. As 4-E5 is tagged at its N-terminus with the HA epitope, co-immunoprecipitation experiments with mAb HA.11 raised against the HA epitope were performed. mAb HA.11 co-precipitated 4-E5 and HC, although not as effectively as Ab 274, (Figure 5.3 C, lane 2) but did not co-precipitate HC with 4-E5T (Figure 5.3 C, lane 3). These data indicate that the interaction between 4-E5 and HC takes place via the C-terminus of 4-E5. To confirm this, competition experiments with a well characterised peptide were performed. The peptide sequence corresponded to the C-terminus of 4-E5, and its ability to compete with 4-E5 had been validated in immunocyto- and immunohisto-staining experiment (Pennie *et al.* 1993; Anderson *et al.* 1997; Araibi *et al.* 2004). The peptide did successfully compete with 4-E5 in co-immunoprecipitation experiments (Figure 5.3 B, lane 11); its addition to the reaction prevented the interaction between 4-E5 and N*01301, proving conclusively that the C-terminus of 4-E5 interacts with MHC class I HC.

Figure 5.3



Lanes: 1, input N*01301
2, N*01301+Ab
3, N*01301+ E5+Ab
4, input E5
5, N*01301+N17Y+Ab
6, N17Yinput

Lanes: 7, N*01301+N17A+Ab
8, N17A input
9, N*01301+E5T+Ab
10, E5T input
11, N*01301+E5+peptide

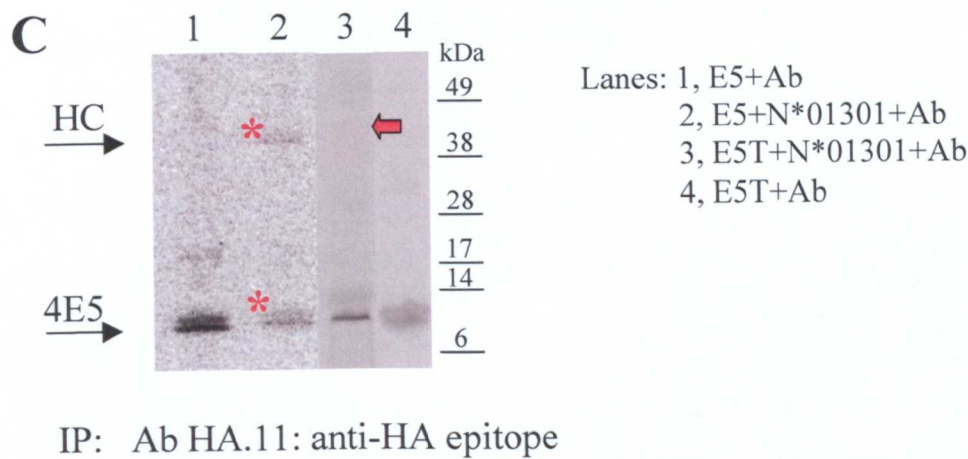


Figure 5.3 BPV E5 and MHC class I HC interact via the C-terminus domain of E5.

For ease of description, 4-E5 wt will be referred to as E5 and 4-E5T as E5T. The numbers on the right represent the molecular weights marker in kilodalton.

A) *In vitro* co-immunoprecipitation with Ab 274

³⁵S-labelled *in vitro* transcribed/translated E5, N17Y or N17A, and N*01301 HC were immunoprecipitated with Ab 274, against the C-terminus of E5; the precipitate was run in a PAGE gel and the gel exposed on a screen on a Storm 840 apparatus. Note that lanes 1-3 and lanes 4-9 were run in separate gels and aligned together for ease of comparison. The red stars indicate the interaction between N*01301 HC with either E5 wt or its mutant forms N17Y or N17A (experiment done by myself from data published in Marchetti *et al.* 2006).

B) *In vitro* co-immunoprecipitation and competition with mAb IL-A88

³⁵S-labelled *in vitro* transcribed/translated E5, N17Y, N17A or E5T and N*01301 HC were immunoprecipitated with mAb IL-A88 against bovine MHC class I HC and processed as in A. E5, N17Y and N17A interact with HC (red stars), whereas E5T, deleted in the C-terminus domain does not (red arrow lane 9). The C-terminus peptide prevents E5 binding to N*01301 HC (red arrow lane 11). Note that lanes 1-4, 5-10 and 11 were run in a separate gel and aligned with the other lanes for ease of comparison (experiment done by myself from data published in Marchetti *et al.* 2006).

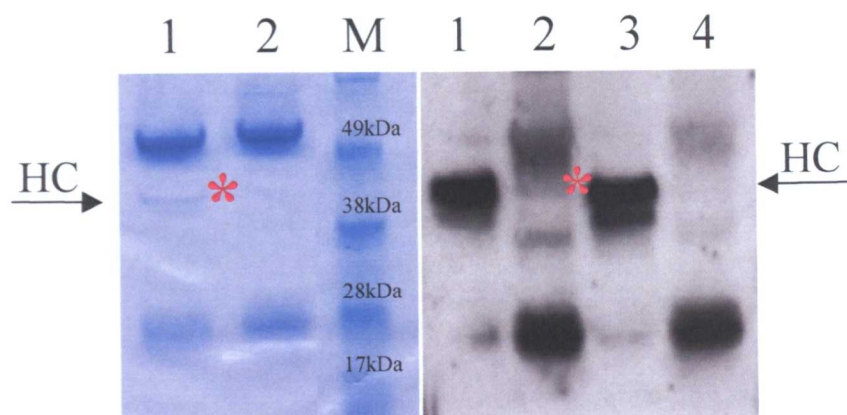
C) *In vitro* co-immunoprecipitation with mAb HA.11

³⁵S-labelled *in vitro* transcribed/translated E5 or E5T and HC were immunoprecipitated with mAb HA.11 against the HA epitope tag of E5 and E5T and processed as in A. Note that lanes 1-2 and 3-4 were run in a separate gel and aligned with the other lanes for ease of comparison. The red stars indicate the interaction

between N*01301 HC with E5 wt. E5T does not interact with N*01301 HC (red arrow lane 3) (experiment done by myself from data published in Marchetti *et al.* 2006).

5.4 *In vivo* interaction between BPV-4 E5 and MHC class I heavy chain

To confirm that 4-E5 and HC interact also in cells, co-immunoprecipitation experiments were performed in no-E5 cells and in 4-E5 cells. Both cell lines were treated with IFN and MG132 (as described in section 4.2 and 4.3) to bring the amount of heavy chain in 4-E5 cells to detectable levels. Protein lysates from both cell lines were incubated with Ab 274 and the immunoprecipitates were run on a gel and immunoblotted with mAb IL-A88. A band of the appropriate MW for HC (approximately 45 kDa) was visible in a Coomassie blue stained gel in the immunoprecipitate from 4-E5 cells, which was not visible in the immunoprecipitate from no-E5 cells (Figure 5.4). This band reacted with mAb IL-A88 and corresponded to bona fide HC detected by direct immunoblotting of protein lysates (Figure 5.4). Despite the combined IFN+MG132 treatment, the HC band co-precipitated with 4-E5 is faint because the treatment does not increase expression of 4-E5 (Figure 4.2 C) and 4-E5 is expressed at almost undetectable levels (O'Brien *et al.* 1999; O'Brien *et al.* 2001); therefore the amount of 4-E5 is limiting for the amount of bound HC. However, the reactivity with mAb IL-A88 specific for bovine HC seen only in 4-E5-expressing cells, the correspondence of the band with bona fide HC and the results obtained *in vitro*, establish the identity of this band as MHC class I HC and confirm that the interaction between 4-E5 and HC takes place also in cells.



IP: Ab 274; IB: mAb IL-A88

Left panel: Coomassie blue stained gel

Lanes: 1, co-IP from 4-E5 cells

2, co-IP from no-E5 cells

M, marker

Right panel: immunoblot

Lanes: 1, protein lysate from 4-E5 cells

2, co-IP from 4-E5 cells

3, protein lysate from no-E5 cells

4, co-IP from no-E5 cells

Figure 5.4 BPV-4 E5 and MHC I HC interact *in vivo*

***In vivo* co-immunoprecipitation**

No-E5 cells and 4-E5 cells were treated with γ -IFN and MG132 as described in materials and methods. Cells were lysed in RIPA buffer containing a cocktail of protease inhibitors (Roche, Lewes, UK). 100 μ g of protein lysate were immunoprecipitated with Ab 274. 10 μ g of the immunoprecipitate were run in NuPAGE gels, transferred to nitrocellulose membranes and immunoblotted with mAb IL-A88 as described in Materials and Methods. A parallel gel was stained with Coomassie blue. The red stars indicate the position of HC (experiment done by Mr Ed Dornan a worker in this laboratory from data published in Marchetti *et al.* 2006).

Three conclusions can be reached from these experiments: one, binding of 4-E5 to HC is *necessary* for down-regulation of MHC class I as shown by 4-E5T, but not *sufficient* as shown by N17Y; two, the nature of residue 17 is not important for the interaction with HC, and three, the C-terminus of 4-E5 is responsible for the interaction between 4-E5 and HC (Table 5.3).

Cell type	Cell transformation	16K interaction (<i>in vitro</i>)	GJIC down regulation	MHC I down regulation	MHC I HC interaction (<i>in vitro</i>)	MHC I HC interaction (<i>in vivo</i>)
4-E5 wt	+	+	+	+	+	+
N17Y	-	+	-	-	+	nd
N17A	++	+	+	+	+	nd
4-E5T	-	+	-	-	-	nd

Table 5.3 Summary of characteristics of 4-E5 and its mutants

16K: 16K ductin/subunitc, a component of the gap junction and of the V0 sector of the vacuolar H⁺-ATPase (Finbow *et al.* 1991; Goldstein *et al.* 1991; Conrad *et al.* 1993; Faccini *et al.* 1996). GJIC: gap junction intracellular communication. nd: not done. The + and – represent the extent of expression of the characteristic.

5.5 *In vitro* transcription translation and immunoprecipitation of BPV-4 E5 and putative non-classical MHC I heavy chain

While classical MHC molecules (HLA-A/B) are the main presenters of antigenic peptides to cytotoxic T lymphocytes (CTL) cells, HLA-C and non-classical HLA/MHC, such as HLA-E or -G, inhibit natural killer (NK) cell-mediated lysis by

interacting with inhibitory NK receptors (Lee *et al.* 1998; Wang *et al.* 1998; Braud *et al.* 1999b). Certain viral proteins, including HIV Nef and the US3/UL40 proteins of CMV, have evolved to downregulate selectively HLA-A and -B, the main presenters of peptides to CTLs, but not HLA-C or non-classical HLA-E (Ahn *et al.* 1996; Tomasec *et al.* 2000; Furman *et al.* 2002) and are therefore capable of avoiding both CTL and NK cell killing (Cohen *et al.* 1999). Human papillomavirus type 16 E5 (HPV-16 E5) selectively down regulates surface expression of HLA-A and HLA-B, which present viral peptides to MHC class I-restricted cytotoxic T lymphocytes but not the NK cell inhibitory ligands HLA-C and non-classical HLA-E (Ashrafi *et al.* 2005).

*N*50001* (formerly known as GeneX or HD59; (Ellis *et al.* 1996) appears to be a bovine non-classical class I gene due to a number of characteristics: it is relatively non-polymorphic, it is prone to alternative splicing, it has an early stop codon in the cytoplasmic domain, producing a truncated C-terminus (19 amino acids shorter than *N*01301* HC), it has a number of unusual amino acid substitutions throughout, it has a large deletion in the 3' UTR, and significant differences in the promoter region compared to classical class I genes (Dr. Shirley Ellis personal communication) (Figure 5.5 A).

While classical MHC I HC contains the C-terminus cytoplasmic tail, and is able to be co-immunoprecipitated with the 4-E5 protein (section 5.1), the product of the bovine non-classical class I gene *N*50001*, lacks this domain.

To determine whether 4-E5 selectively down-regulates classical MHC class I and which HC domain is involved, co-immunoprecipitation experiments were performed.

The *N*50001* HC was already cloned in the pcDNA6-V5-His vector under the control of the T7 promoter (as described in chapter 2, sections 2.1.10 and 2.1.11). 4-E5 and

the N*50001 HC were transcribed/translated *in vitro* in the presence of ³⁵S-methionine and immunoprecipitated as described in Materials and Methods.

The proteins were precipitated with mAb IL-A88 against bovine HC (this antibody is known to work well with classical and non-classical HC; Shirley Ellis personal communication) (Fig 5.5 B,C).

There was little or no precipitate in absence of antibody (Figure 5.5 D, lane 2); mAb IL-A88 precipitated N*50001 HC but not 4-E5 (figure 5.5 D, lane 3, 4). While 4-E5 was efficiently co-immunoprecipitated by mAb IL-A88 with N*01301 HC that contains the cytoplasmic tail (Figure 5.5 D, lane 5), 4-E5 was not co-immunoprecipitated with N*50001 HC that lacks this domain (Figure 5.5 D, lane 6).

To confirm this result, we performed competition experiments between N*01301 HC and 4-E5 with unlabelled N*50001 HC. The proteins were again precipitated with mAb IL-A88. N*50001 HC did not compete with 4-E5 in co-immunoprecipitation experiments when 4-E5, N*01301 HC and N*50001 HC were used together (Figure 5.5 E lane 2); the addition of N*50001 HC to the reaction did not prevent the interaction between 4-E5 and N*01301. It is reasonable to conclude that the BPV-4 E5 and the non-classical MHC I HC do not interact *in vitro*.

As described above, N*50001 lacks the C-terminus intra-cytoplasmic tail of classical MHC class I HC and is 19 amino acids shorter than N*01301 (Figure 5.5 A). Given the absence of interaction between E5 and N*50001 we wondered whether the intra-cytoplasmic domain of class I HC was responsible for binding to E5. A C-terminus truncation mutant of N*01301 HC lacking the final 19 amino acid residues was made (N*01301stop₃₃₉) and used in *in vitro* co-IP experiments with 4-E5. This mutant co-precipitated with 4-E5 (figure 5.5 F lane 3) showing that the interaction between HC

and 4-E5 does not involve the C-terminus intra-cytoplasmic domain of HC. Furthermore, N*01301 HC bound E5 whether tagged with V5-His at its C-terminus, like N*50001, or not (figure 5.5 F lanes 11 and 7), demonstrating that the C-terminus epitope has no effect on the interaction.

It is not possible to analyze the expression or the function of *N*50001* in bovine cells (*in vivo*) because there is no specific antibody for its protein. To study the relationship of BPV-4 E5 with classical and non-classical MHC I, Araibi et al., separately expressed a classical, *N*01301*, and a non-classical, *N*50001*, allele in the mouse mastocytoma P815 cells (Ellis *et al.*, 1999) and subsequently introduced E5 in these cells (Araibi *et al.*, 2006). The lack of *in vitro* interaction between E5 and N*50001 (see fig 5.5 D and E in this thesis) was corroborated by a “pull-down co-immunoprecipitation” experiment between E5 and N*50001 HC in the P815 cells expressing the two proteins (Araibi *et al.* 2006). Moreover, Araibi et al, showed that BPV-4 E5 does not retain the non-classical MHC class I in the Golgi, does not inhibit its transport to the cell surface and does not affect the stability of N*50001 HC (Araibi *et al.*, 2006). Taken together, the results presented above lead to the firm conclusion that E5 does not interfere with the biosynthetic pathway of non-classical N*50001 MHC class I.

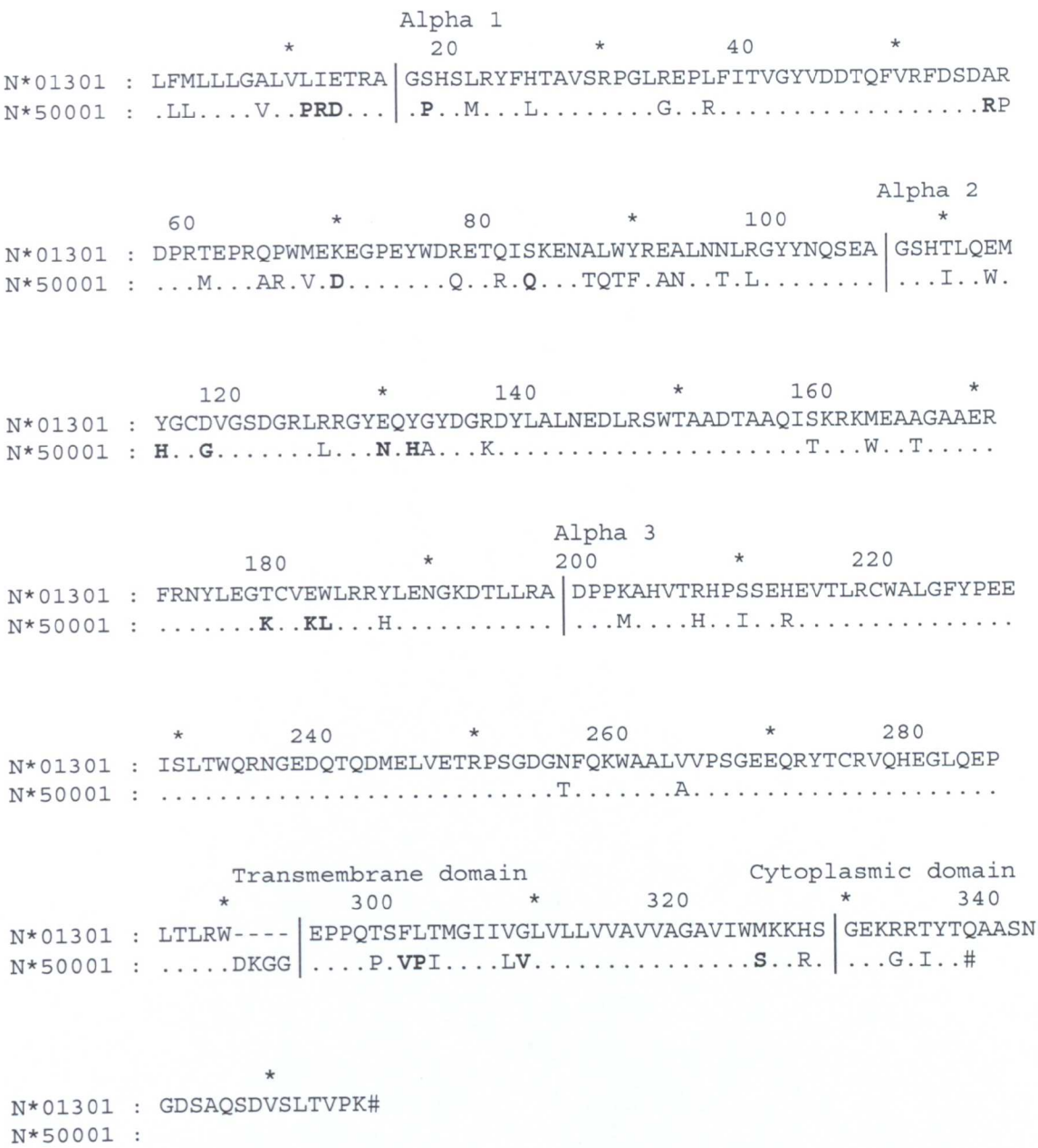


Figure 5.5 A) Alignment of the predicted amino acid sequences of N*01301 and N*50001

Dots indicate identity, # indicates stop codon. Divergent amino acids (bold) are seen only in N*50001 (or other cattle non-classical class I alleles) and are not seen in cattle classical class I alleles (www.ebi.ac.uk/ipd/mhc/bola/). The alignment was achieved with DNASTAR software using the Megalignment program.

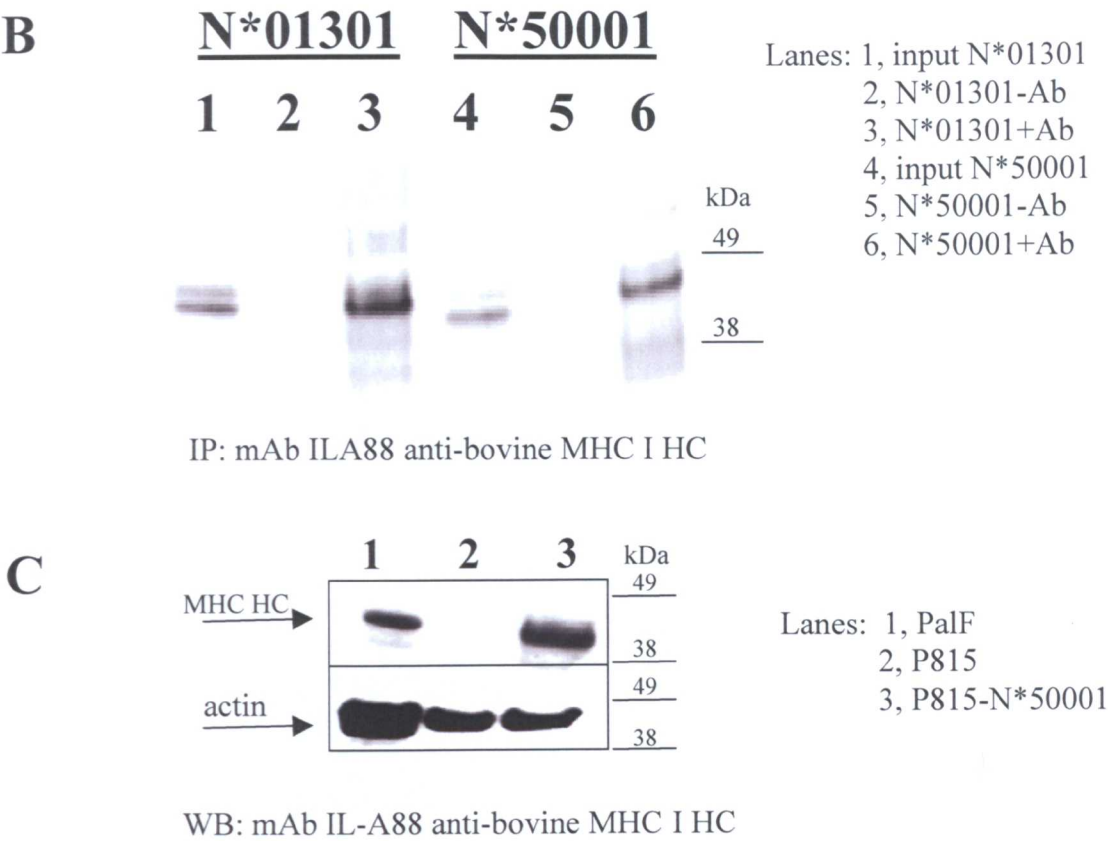


Figure 5.5 mAb IL-A88 which recognises all cattle classical class I genes also recognises N*50001

B) *In vitro* immunoprecipitation

The plasmids pcDNA3 and pcDNA6 encoding N*01301 (classical) and N*50001 (non-classical), respectively were transcribed/translated in presence of ³⁵S-methionine and the products were immunoprecipitated with mAb IL-A88 against bovine MHC class I HC; the precipitates were run in a 4-12% NuPAGE gel and the dried gel exposed on a screen and analysed on a Storm 840 apparatus. mAb IL-A88 recognise N*50001 in immunoprecipitation (experiment done by myself).

C) Western blot analysis

Bovine PalF cells, mouse mastocytoma cells P815 (described in Materials and Methods) or P815 cells expressing N*50001 (P815- N*50001) (described by Ellis *et al.* 1999) were harvested and 10µg protein lysates were immunoblotted with mAb IL-A88 against HC, or mAb AB-1 against actin. mAb IL-A88 recognises HC from bovine PalF cells and from mouse cells stably expressing bovine N*50001 (P815- N*50001) and does not cross react with mouse HC (experiment done by myself).

The numbers on the right in B and C represent the molecular weights marker in kilodalton.

Figure 5.5

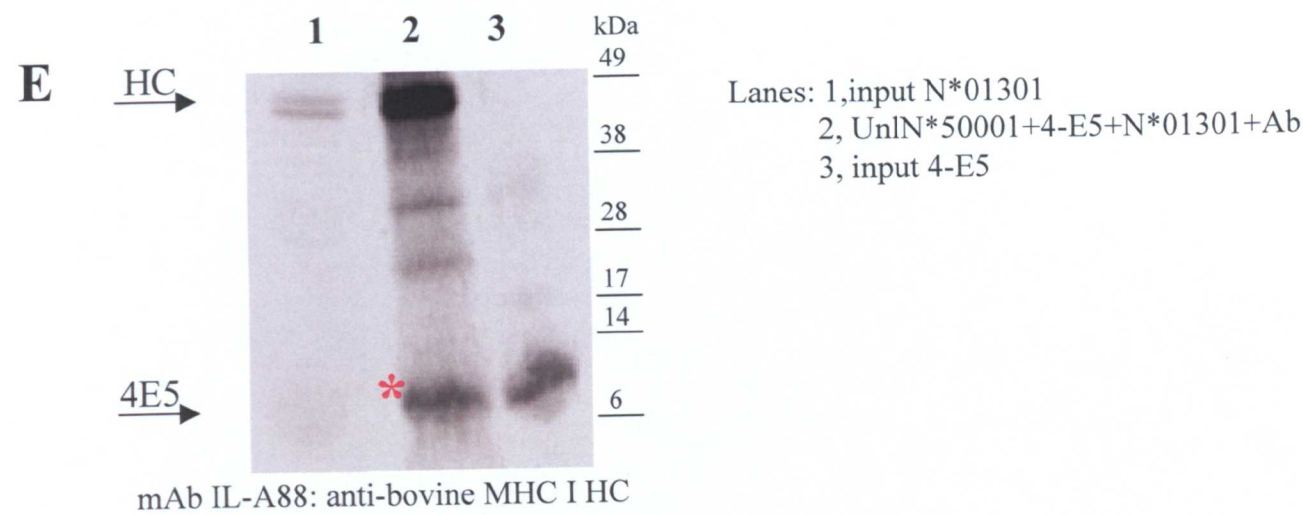
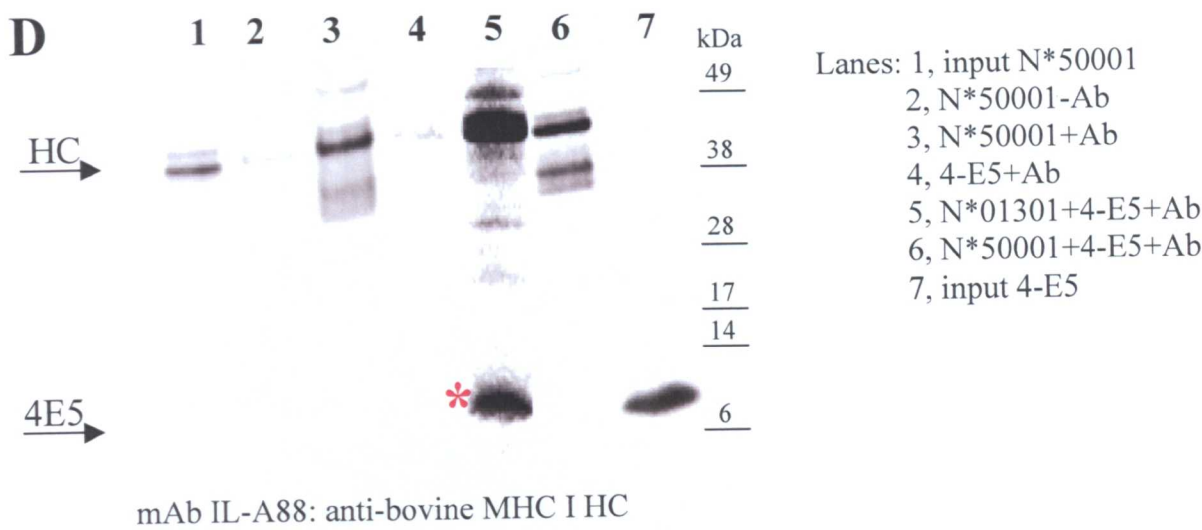


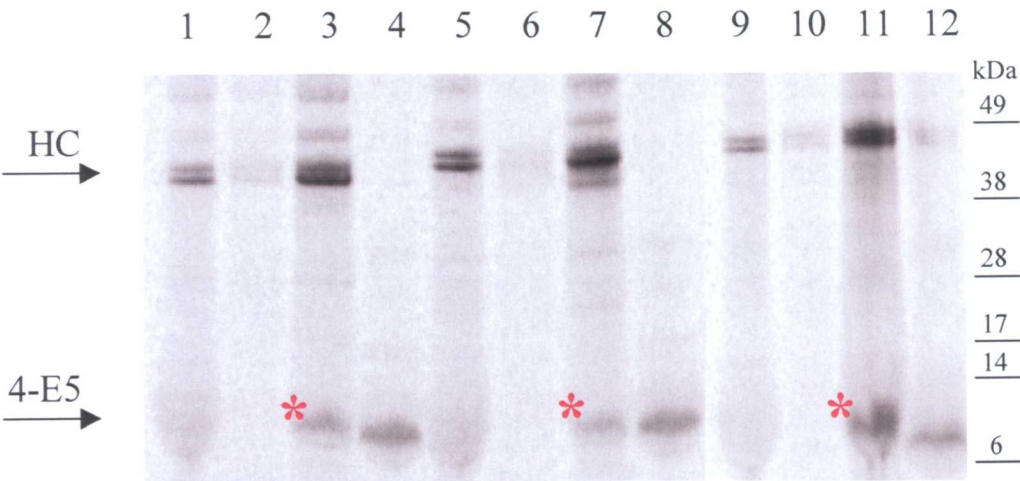
Figure 5.5 BPV-4 E5 and non-classical MHC class I HC do not interact *in vitro*.**D) *In vitro* co-immunoprecipitation**

³⁵S-labelled *in vitro* transcribed/translated 4-E5, N*01301 HC and N*50001 HC were immunoprecipitated with mAb IL-A88 against bovine MHC class I HC; the precipitate was run in a 4-12% NuPAGE gel and the dried gel exposed on a screen and analysed on a Storm 840 apparatus. 4-E5 interacts with classical N*01301 HC (red star, lane 5) but does not interact with non-classical N*50001 HC (red arrow, lane 6). Note that lanes 1-5 and 6-7 were run in a separate gels and aligned with the other lanes for ease of comparison.

E) *In vitro* competition and co-immunoprecipitation

³⁵S-labelled N*01301 HC and 4-E5 and unlabelled N*50001 HC were *in vitro* transcribed/translated and immunoprecipitated with mAb IL-A88. The precipitate was treated as in D. Non-classical N*50001 HC fails to compete with classical N*01301 HC for binding to 4-E5 (red star, lane 2). The numbers on the right in D and E represent the molecular weights marker in kilodalton (experiments done by myself from data published in Araibi *et al.*, 2006)

F



mAb IL-A88: anti-bovine MHC I HC

Lanes: 1, input N*01301stop₃₃₉
2, N*01301stop₃₃₉ +4-E5-Ab
3, N*01301stop₃₃₉ +4-E5 +Ab
4, input 4-E5
5, input N*01301
6, N*01301 +4-E5-Ab

7, N*01301 +4-E5 +Ab
8, input 4-E5
9, input N*01301-V5-His
10, N*01301-V5-His+ 4-E5-Ab
11, N*01301-V5-His+ 4-E5+Ab
12, input 4-E5

Figure 5.5 The intra-cytoplasmic domain of class I HC is not responsible for binding BPV-4 E5

F) *In vitro* co-immunoprecipitation

³⁵S-labelled *in vitro* transcribed/translated 4-E5, N*01301 HC and N*01301stop₃₃₉ HC were immunoprecipitated with mAb IL-A88 against bovine MHC class I HC; the precipitate was run in a 4-12% NuPAGE gel and the dried gel exposed on a screen and analysed on a Storm 840 apparatus. The C-terminus truncated form N*01301stop₃₃₉ still interacts with 4-E5 (red star, lane 3) and N*01301 HC bound 4-E5 whether tagged with V5-His at its C-terminus (red star, lane 11) or not (red star, lane 7). The interaction between HC and 4-E5 does not involve the C-terminus domain of HC. Note that lanes 1-8 and 9-12 were run in separate gels and aligned with the other lanes for ease of comparison. The numbers on the right represent the molecular weights marker in kilodalton (experiment done by myself).

CHAPTER 6: PV E5 proteins interact *in vitro* with human and equine major histocompatibility class I heavy chains: common way to escape immunosurveillance ?

6.1 *In vitro* immunoprecipitation between classical HLA and HPV-16 E5

HPV-16 E5 also prevents the transport of classical MHC (HLA) class I complexes to the cell surface and retains it in the Golgi apparatus (Ashrafi *et al.* 2005). It was hypothesised that, like BPV-4 E5, HPV-16 E5 could retain HLA class I HC in the Golgi apparatus by physically interacting with these molecules. To investigate if such interaction exists, *in vitro* transcription/translation and co-immunoprecipitation experiments were performed. First, different HLA haplotypes were cloned in commercial cloning plasmids suitable for transcription/translation assay (generation of constructs described in chapter 2, sections 2.1.10 and 2.1.11). The HPV-16 E5 protein was already tagged at its N-terminus with an HA epitope and cloned in a plasmid (pCI-neo) under the control of the T7 promoter (details of construct in chapter 2, section 2.1.10 and 2.1.11). Its efficiency of translation was assessed by *in vitro* transcription/translation assay as described in Materials and Methods (Fig. 6.1 A). HPV-16 E5 and the human HLA HCs were separately transcribed/translated *in vitro* in the presence of ^{35}S -methionine and then kept separately or mixed together. The proteins were precipitated either with mAb HC10, specific for the human HLA class I HC or mAb HA.11 raised against the HA epitope. The mAb HC10 precipitated HLA-A1, A2 and B8 HCs but not 16-E5 (Figure 6.1 B, lanes 3,11,19,5,13,21 upper panel), and the mAb HA.11 precipitated 16-E5 but little or no HCs (Figure 6.1 B, lanes 5,13,21,3,11,19 bottom panel). However, when the two proteins were mixed together, they co-precipitated with either antibody (Figure 6.1 B, lanes 7,15,23 upper

and bottom panel), indicating that 16-E5 and the HCs from different haplotypes interact, at least *in vitro* (Ashrafi *et al.* 2006b). These results shown that the interaction is not restricted to certain HLA alleles.

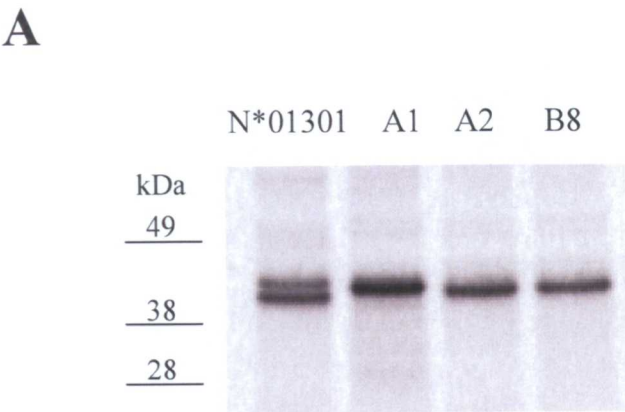


Figure 6.1 HPV-16 E5 and classical HLA class I HC interact *in vitro*

A) Transcription/translation assay

N*01301, HLA-A1, HLA-A2 and HLA-B8 were *in vitro* transcribed/translated in presence of ³⁵S-methionine. The produced proteins were run on a 4-12% NuPAGE gel and the dried gel exposed on a screen and analysed on a Storm 840 apparatus. The bovine N*01301 HC was run alongside the human HCs as a control of transcription/translation reaction and as a comparison for the molecular weight of the resultant proteins. The numbers on the left represent the molecular weights marker in kilodalton (experiment done by myself).

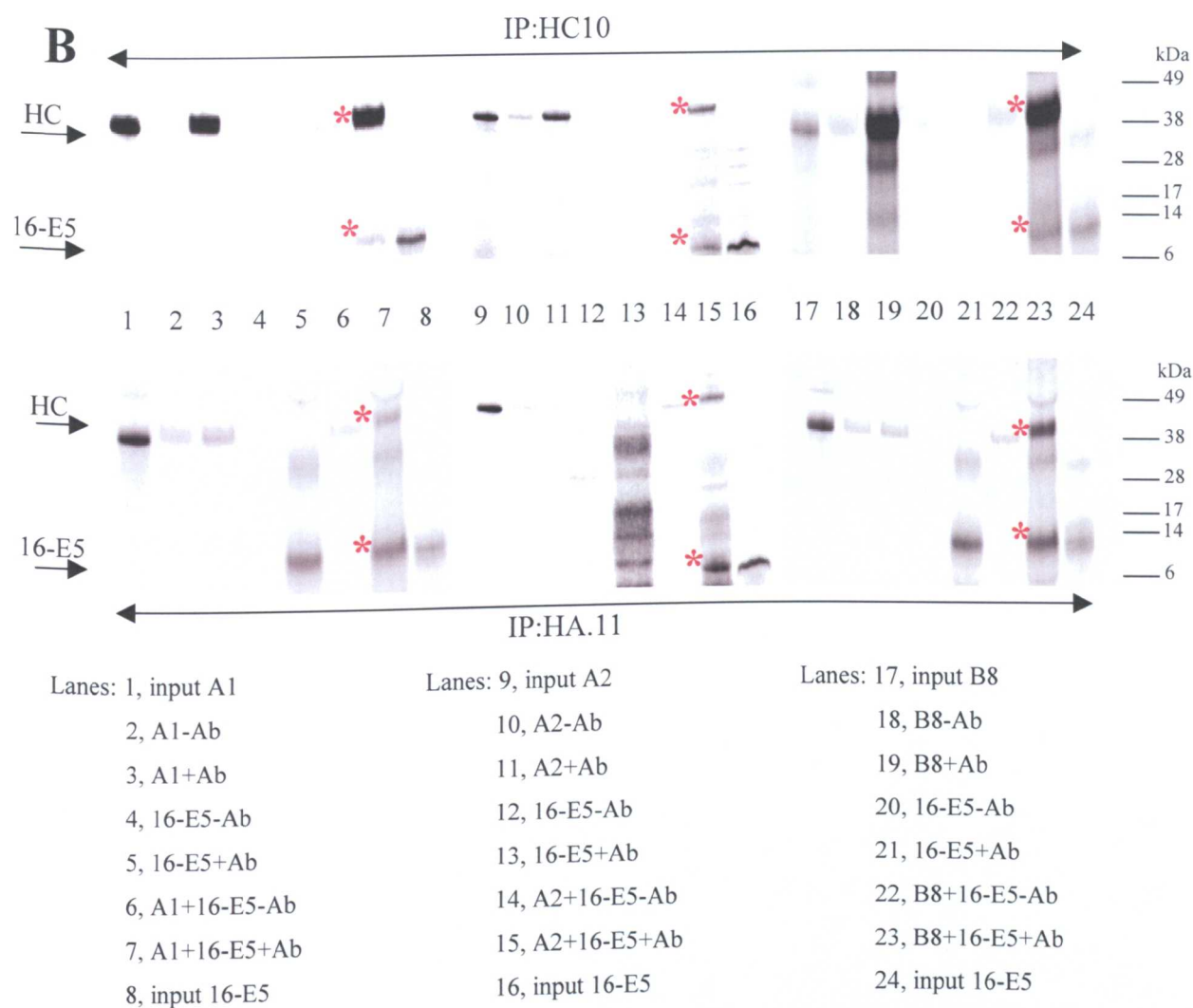


Figure 6.1 HPV-16 E5 and classical HLA class I HC interact *in vitro*

B) *In vitro* co-immunoprecipitation

³⁵S-labelled *in vitro* transcribed/translated 16-E5, A1, A2 and B8 were immunoprecipitated with mAb HC10 specific for the human HLA class I heavy chain (upper gels) or mAb HA.11 raised against the HA epitope (bottom gels). The precipitates were run on a 4-12% NuPAGE gel as above. The red stars indicate the interaction between 16-E5 and either HLA-A1 (lane 7), HLA-A2 (lane 15) or HLA-B8 (lane 23). Note that lanes 1-8, 9-16 and 17-24 were run in separate gels and aligned with the other lanes for ease of comparison. The numbers on the right represent the molecular weights marker in kilodalton (experiment done by myself).

6.2 BPV and equine sarcoids

The equine sarcoid, a locally aggressive, fibroblastic skin tumour, is the most common dermatological neoplasm reported in horses. Although the disease is not metastatic, the lesions rarely regress and if left untreated can become aggressive. Despite the strict species-specificity of PVs, it has been shown that BPV-1 and less frequently BPV-2 are associated with the pathogenesis of sarcoid disease (Lancaster *et al.* 1977; Gorman 1985; Nasir and Reid 1999; Martens *et al.* 2001). However, there are numerous differences between BPV-1 or BPV-2 infection in cattle and in horses. Infection in cattle results in benign cutaneous fibropapillomas, productive for infectious progeny virus. Bovine papillomas regress in response to an appropriate effector immune response. Although the precise mechanisms are not clear, a cell mediated immune response is the major contributor to the regression of papillomas (Okabayashi *et al.* 1991; Coleman *et al.* 1994; Knowles *et al.* 1996). In contrast, BPV-1 or BPV-2 infection in horses results in sarcoids, non permissive for virus production (Gorman 1985), locally aggressive and non-regressing (Ragland 1970). Although equine sarcoids are not permissive for virus production, the BPV-1 or BPV-2 major transforming protein, E5, is expressed in over 75% of sarcoids (Nasir and Reid 1999; Carr *et al.* 2001b; Chambers *et al.* 2003b). It is still not known how E5 contributes to sarcoid pathogenesis. One hypothesis could be that expression of E5 in sarcoids is sufficient to downregulate the equine leucocyte antigen (ELA/MHC), likely resulting in immune evasion as it has been hypothesized for BPV-4 E5 and bovine classical MHC class I and HPV-16 E5 and classical HLA.

6.2.1 *In vitro* immunoprecipitation between ELA HC and BPV-1 E5

To investigate the possibility of an interaction between the ELA HC and the BPV-1 E5 *in vitro* immunoprecipitation were performed. We received plasmids encoding two classical ELA HC: EqB2 and EqB4 (kind gift of Dr. Shirley Ellis). The full length BPV-1 E5 protein was cloned in a plasmid suitable for transcription translation assay (as described in chapter 2, section 2.1.10 and 2.1.11). The efficiency of translation was assessed by *in vitro* transcription/translation assay (Fig. 6.2.1 A and B lane 1). BPV-1 E5 and the ELA HCs were separately transcribed/translated *in vitro* in the presence of ^{35}S -methionine and then kept separately or mixed together. The proteins were precipitated with either mAb MAC291 or mAb H58A, specific for the equine class I HC. To assess the reactivity of the two available mAb MAC291 and mAb H58A a preliminary immunoprecipitation was performed. The two mAb showed different reactivities with mAb MAC291 reacting better with the EqB2 class I HC and the mAb H58A with EqB4 class I HC (figure 6.2.1 C). Taking account of this result, mAb MAC291 and mAb H58A were chosen to immunoprecipitate EqB2 HC and EqB4 HC, respectively.

The mAb MAC 291 immunoprecipitate EqB2 HC but not 1-E5wt (figure 6.2.1 D lanes 3,4 upper gel) and mAb H58A immunoprecipitate EqB4 but not 1-E5wt (figure 6.2.1 D lanes 3,4 bottom gel). However, when the two proteins were mixed together, they co-precipitated with the appropriate antibody (figure 6.2.1 D lanes 6 upper and bottom gels) indicating that BPV-1 E5wt interacts with equine class I HC at least *in vitro*, and this interaction can take place with two different equine alleles.

To further investigate if this *in vitro* interaction occurs via the C-terminus of the BPV-1E5 protein as shown for the BPV-4 E5 (section 5.3), a truncated form of BPV-1 E5 with a stop codon in position 32 (1-E5T) was generated by PCR and cloned in a

suitable plasmid for *in vitro* transcription/translation (construct details in chapter 2, sections 2.1.10 and 2.1.11). The efficiency of translation of 1-E5T was assessed by *in vitro* transcription/translation assay (Fig. 6.2.1 B lane 2). Then, co-immunoprecipitations between the truncated 1-E5T which lacks the C-terminus domain and the equine HCs (B2, B4) were performed. The 1-E5wt was co-precipitated with HC by mAb MAC291 or mAb H58A (figure 6.2.1 D, lane 6 upper and bottom gels), while 1-E5T was not co-precipitated either with EqB2 HC or EqB4 HC (figure 6.2.1 E, lanes 5 and 9).

To confirm this result, competition experiments between EqB2 HC or EqB4 HC and 1-E5 with either unlabelled 1-E5T or unlabelled 1-E5 were performed. As expected, the full length unlabelled 1-E5 competed with itself preventing the interaction of labelled E5 with both EqB2HC and EqB4 HC (Figure 6.2.1 F lanes 2 and 5), whereas the unlabelled 1-E5T protein did not compete with 1-E5 (Figure 6.2.1 G lanes 2 and 5) and the addition of 1-E5T to the reaction did not prevent the interaction between 1-E5 and EqB2 HC or EqB4 HC.

These data indicate, conclusively, that the interaction between 1-E5 and equine HCs takes place via the C-terminus of 1-E5, as shown for 4-E5.

These results show a common behaviour between the E5 proteins of BPV-1 and BPV-4 at least *in vitro*.

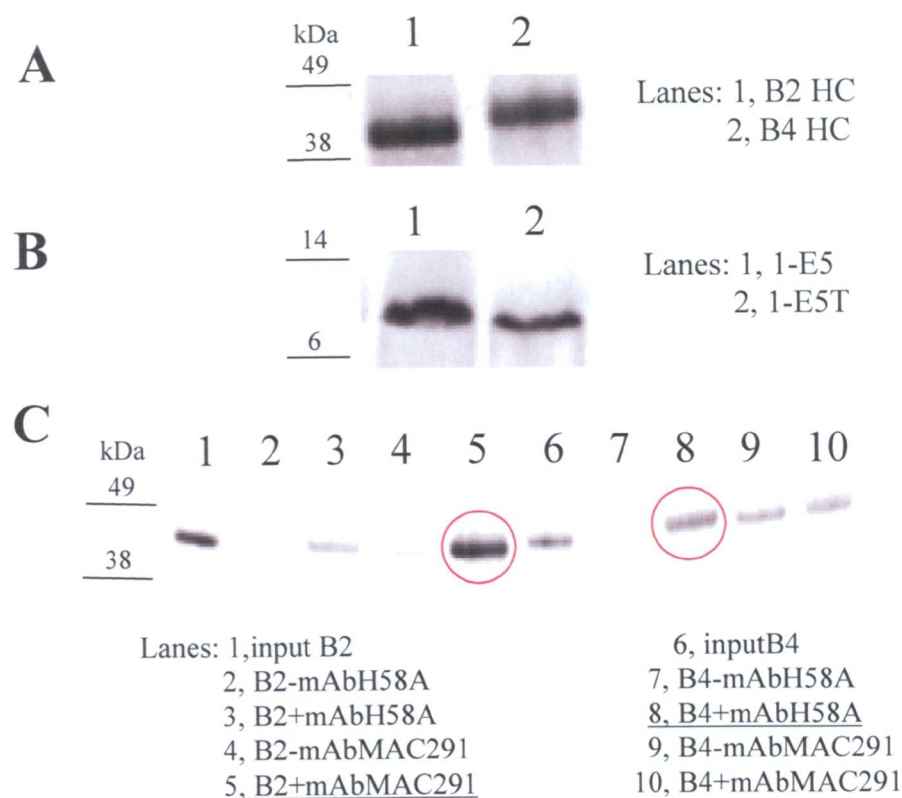


Figure 6.2.1 Equine HC transcription/translation assay and control immunoprecipitation

Equine leucocyte antigen EqB2 (B2) HC, EqB4 (B4) HC (A), BPV-1E5wt (1-E5) and BPV-1E5T (1-E5T) (B) were *in vitro* transcribed/translated in presence of ³⁵S-methionine. The produced proteins were run on a 4-12% NuPAGE gel and the dried gel exposed on a screen and analysed on a Storm 840 apparatus to assess the efficiency of translation. (C) **Equine HC control immunoprecipitation** ³⁵S-labelled *in vitro* transcribed/translated EqB2 HC and EqB4 HC were immunoprecipitated with mAb MAC291 and mAb H58A specific for the equine class I heavy chain. The precipitated were run on a 4-12% NuPAGE gel and treated as above. The mAb MAC291 reacts better with EqB2 (red circle, lane 5 underlined in figure text) and mAb H58A reacts better with EqB4 (red circle, lane 8 underlined in figure text). The numbers on the left represent the molecular weights marker in kilodalton (experiments done by myself).

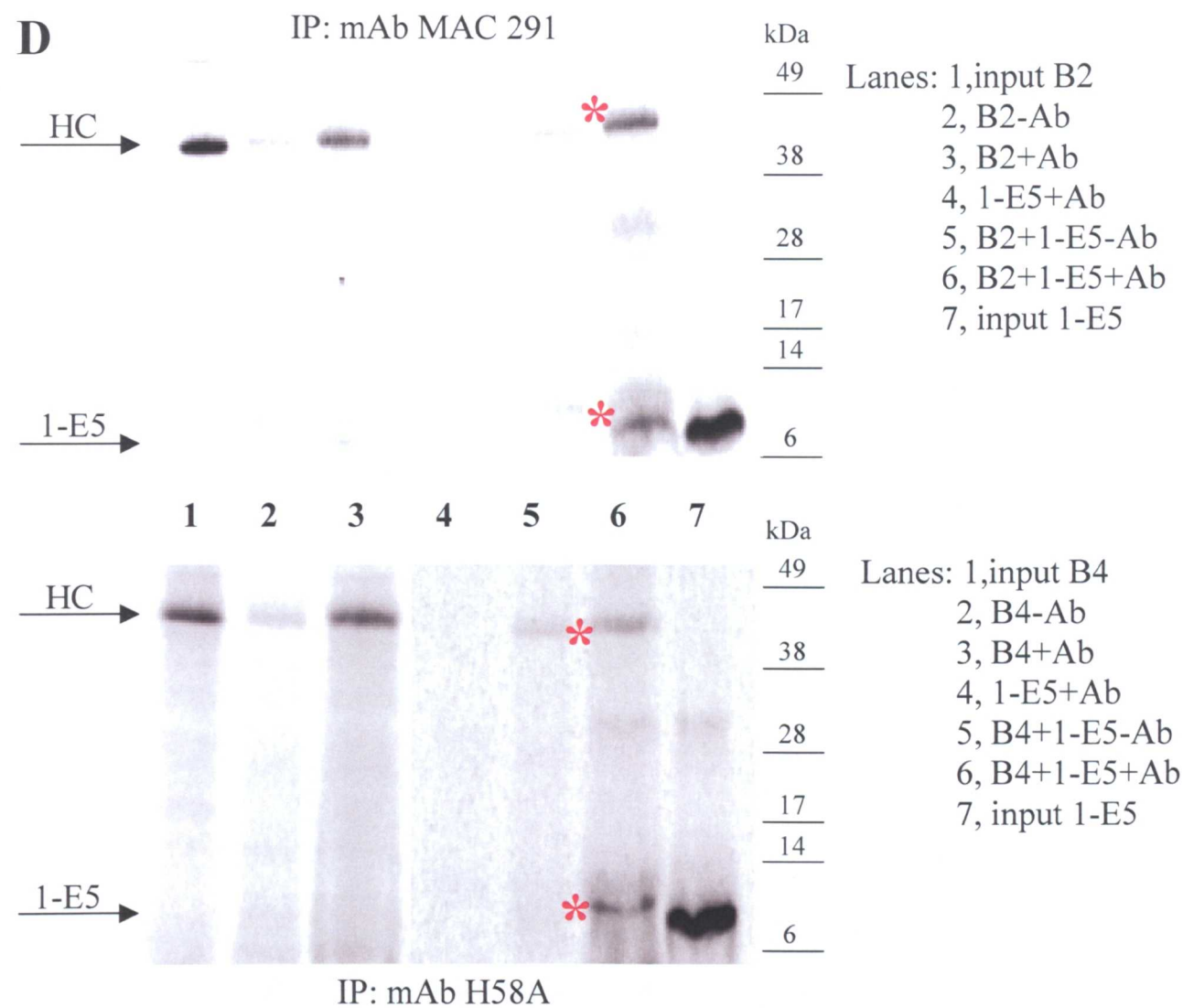


Figure 6.2.1 *In vitro* co-immunoprecipitation between equine HCs and BPV-1E5

D) ³⁵S-labelled *in vitro* transcribed/translated EqB2 (B2), EqB4 (B4) and BPV-1E5wt (1-E5) were immunoprecipitated with mAb MAC291 (upper gel) and mAb H58A (bottom gel) specific for the equine class I heavy chain. The precipitates were run on a 4-12% NuPAGE gel and the dried gel exposed on a screen and analysed on a Storm 840 apparatus. The red stars indicate the interaction between 1-E5 wt and either B2 (lane 6, upper gel) or B4 (lane 6 bottom gel). Note that lanes 1-3, 4-5 of the bottom picture were run in separate gels and aligned with the other lanes for ease of comparison. The numbers on the right represent the molecular weights marker in kilodalton (experiments done by myself).

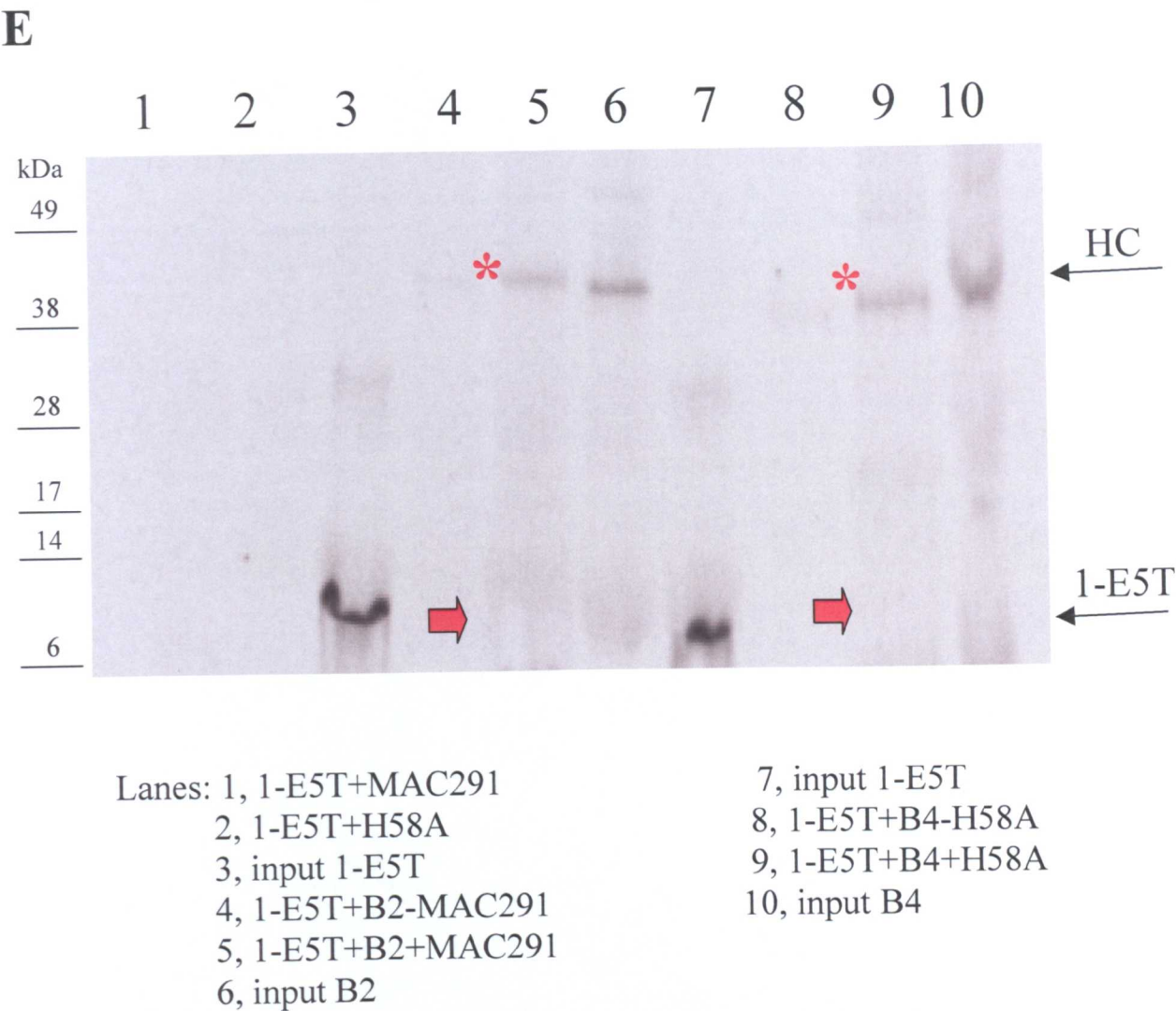
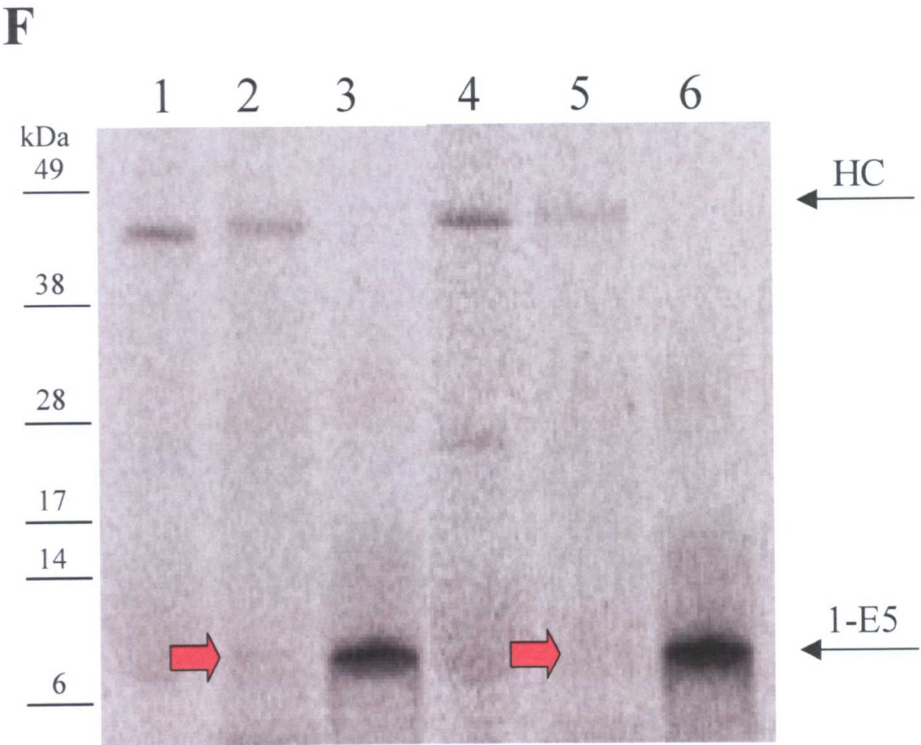


Figure 6.2.1 *In vitro* co-immunoprecipitation between equine HCs and BPV-1E5T

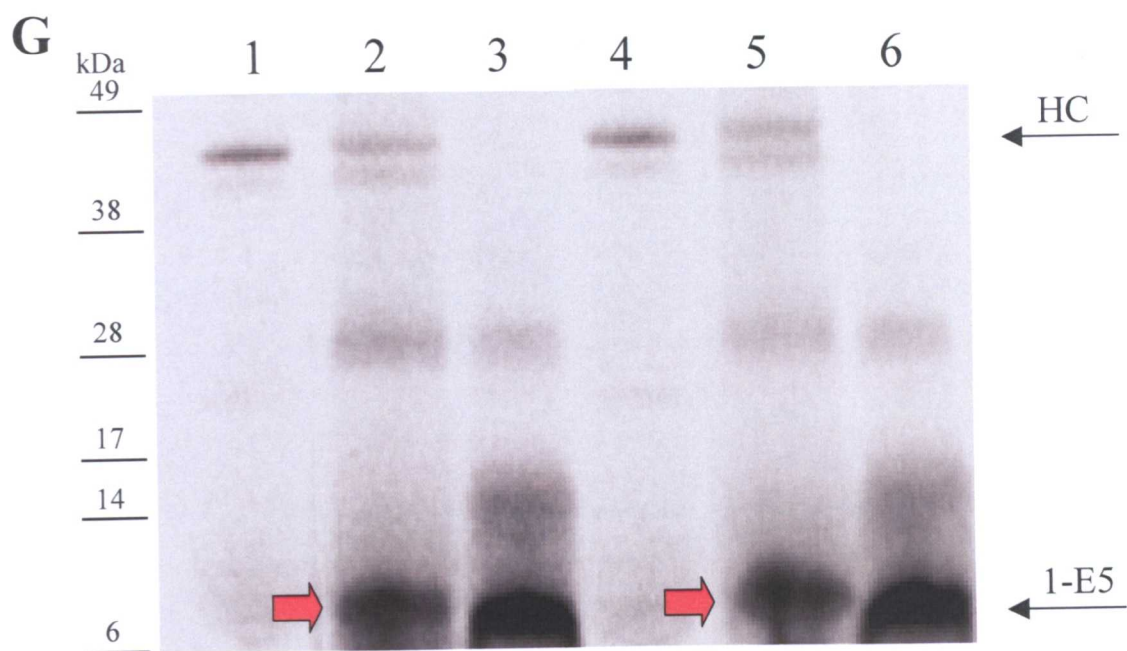
E) ³⁵S-labelled *in vitro* transcribed/translated EqB2 (B2), EqB4 (B4) and BPV-1E5T (1-E5T) were immunoprecipitated with mAb MAC291 or mAb H58A specific for the equine class I heavy chain. The precipitates were run on a 4-12% NuPAGE gel and the dried gel exposed on a screen and analysed on a Storm 840 apparatus. 1-E5T does not interact (red arrows) either with B2 (red star, lane 5) or B4 (red star, lane 9). Note that lanes 1-7 and 8-10 were run in separate gels and aligned with the other lanes for ease of comparison. The numbers on the left represent the molecular weights marker in kilodalton (experiment done by myself).



Lanes: 1, input B2
2, B2+unl1-E5+1-E5+ MAC291
3, input 1-E5
4, input B4
5, B4+unl1-E5+1-E5+ H58A
6, input 1-E5

Figure 6.2.1 The BPV-1 E5 protein compete with itself in co-immunoprecipitation experiment

F) ³⁵S-labelled *in vitro* transcribed/translated EqB2 (B2),EqB4 (B4), BPV-1E5wt (1-E5) and unlabelled 1-E5 (unl1-E5) were immunoprecipitated with mAb MAC291 or mAb H58A specific for the equine class I heavy chain. The unlabelled 1-E5 protein prevent its interaction with the equine HCs (red arrows, lanes 2 and 5). The precipitated were run on a 4-12% NuPAGE gel and the dried gel exposed on a screen and analysed on a Storm 840 apparatus. The numbers on the left represent the molecular weights marker in kilodalton (experiment done by myself).



Lanes : 1, input B2
2, 1-E5+unl1-E5T+B2+ MAC291
3, input 1-E5
4, input B4
5, 1-E5+unl1-E5T+B4+H58A
6, input 1-E5

Figure 6.2.1 The BPV-1 E5T protein does not compete with BPV-1 E5 in co-immunoprecipitation experiment

G) ³⁵S-labelled *in vitro* transcribed/translated EqB2 (B2), EqB4 (B4), BPV-1E5wt (1-E5) and unlabelled 1-E5T (unl1-E5T) were immunoprecipitated with mAb MAC291 or mAb H58A specific for the equine class I heavy chain. The 1-E5T protein did not prevent the interaction between 1-E5 and the equine HCs (red arrows, lanes 2 and 5). The precipitated were run on a 4-12% NuPAGE gel and the dried gel exposed on a screen and analysed on a Storm 840 apparatus. Note that lanes 1-3 and 4-6 were run in separate gels and aligned with the other lanes for ease of comparison. The numbers on the left represent the molecular weights marker in kilodalton (experiment done by myself).

CHAPTER 7: DISCUSSION

7.1 Introduction

During viral infection or malignant transformation, a spectrum of peptides derived intracellularly from viral or transformation-associated antigens is bound to MHC I molecules in the endoplasmic reticulum (ER). Following transport from the ER through the Golgi apparatus (GA) to the cell surface, the MHC class I/peptide complexes are presented to virus-specific cytotoxic T lymphocytes (CTL), signalling virus infection and enabling killing of the infected or tumour cell.

MHC I molecules therefore play a crucial role in immune recognition and clearance of these types of cell. Viruses need to escape immunosurveillance by the host to establish infection and to generate new infectious progeny.

The importance of MHC class I molecules in virus clearance has been demonstrated by the identification of many mechanisms of interference with the MHC class I pathway evolved by viruses, starting with prevention of peptide processing, as in the case of EBV, HCMV and HIV (Klenerman *et al.* 1994; Bertoletti *et al.* 1994; Gilbert *et al.* 1996; Tellam *et al.* 2001) followed by interference with antigenic peptide transport via TAP complex, as in the case of HSV, HCMV, HPV-6 and HPV-11 (Ahn *et al.* 1997; Lehner *et al.* 1997; Galocha *et al.* 1997; Vambutas *et al.* 2001) and finally inhibition of MHC class I surface expression, as in the case of Ad, HCMV, MCMV, HIV and HTLV-1 (Bennett *et al.* 1999; Johnson *et al.* 2001; Kasper *et al.* 2005; Williams *et al.* 2005; Piguet 2005). In each of these strategies, the outcome is the failure by the infected cell to present viral peptide antigens to effector T cells and hence avoidance of detection and destruction by the host immune system.

Papillomaviruses are poorly immunogenic. Even in immunocompetent hosts, whether animals or humans, PV persist for a significant period of time, usually spanning several months, before activation of the host immune system. This lack of recognition suggests the host immune system is unaware of, or disabled by, PV infection.

The ability of the virus to persist has been attributed to the nature of the virus life cycle, which may prevent the immune cells having access to the viral proteins. In fact these proteins are expressed in the suprabasal layers of the epithelium, i.e. in cells not readily exposed to the immune system, and moreover are produced in very low amounts, often below the threshold required for the activation of immune cells, thus leading to tolerance (Tindle 2002).

Together with E6 and E7, E5 is a transforming protein of PV. While E6 and E7 are the main oncoproteins of mucosal human papillomavirus (HPVs) (Munger *et al.* 2001; Mantovani *et al.* 2001), E5 is the major oncoprotein of bovine papillomaviruses (BPVs), particularly of BPV-1 (DiMaio and Mattoon 2001). The E5 protein is a small hydrophobic peptide (from 83 amino acid residues in HPV-16 to 42 residues in BPV-4). Data suggest that BPV E5 is a type II membrane protein with a high hydrophobic α -helical trans-membrane domain and an hydrophilic C-terminus domain oriented inside the Golgi lumen (Surti *et al.* 1998) whereas HPV-16 E5 is composed of three hydrophobic domains that are capable of spanning a membrane three times (Bubb *et al.* 1988) or may exist in a β -hairpin conformation, in which the N- and C- terminus domains exist within the membrane, connected by a loop the central portion of which is exposed on the cytoplasmic or luminal surface, but its orientation in membranes is still unknown (Alonso *et al.* 2002). It is expressed during the early stages of infection in the deep layers of the infected epithelium and its expression is extinguished as the lesion progresses (Burnett *et al.* 1992; Anderson *et al.* 1997; Chang *et al.* 2001).

In agreement with its hydrophobic nature, E5 is localized in the endomembrane compartments of the endoplasmic reticulum and Golgi apparatus of the host cell (Burkhardt *et al.* 1989; Pennie *et al.* 1993). Although there is evidence that in organotypic raft culture E5 can support cell cycle progression and activate late viral functions upon epithelial differentiation (Fehrmann and Laimins 2003), its function *in vivo* is not known but *in vitro* cell transformation is brought about by the activation of several kinases, from growth factor receptors to cyclins-cdks (Morgan and Campo 2000).

E5 interacts physically with the cellular protein 16k ductin/subunit c, a component of the gap junction and of the V0 sector of the vacuolar H⁺-ATPase (Goldstein *et al.* 1991; Conrad *et al.* 1993; Finbow *et al.* 1995; Faccini *et al.* 1996). This physical interaction has been deemed responsible for the down-regulation of gap junction communication (Oelze *et al.* 1995; Faccini *et al.* 1996; Ashrafi *et al.* 2000) and for the lack of acidification of endosomes and GA (Straight *et al.* 1995; Schapiro *et al.* 2000). The inhibition of acidification of the endomembrane components has been attributed to a malfunction of the H⁺-ATPase (Briggs *et al.* 2001), although lack of acidification of late endosomes or inhibition of the vacuolar proton pump are not always observed (Thomsen *et al.* 2000; Ashby *et al.* 2001).

Previous data from this laboratory have shown that transformed bovine cells expressing BPV-1 E5 or BPV-4 E5 are highly vacuolated and have an enlarged and often disrupted GA (Ashrafi *et al.* 2000; Ashrafi *et al.* 2002), and this morphology has been attributed to the interaction of E5 and 16k subunit c. In these cells, E5 proteins, including E5 from HPV-16, cause down-regulation of the major histocompatibility class I (MHC I) complex (Ashrafi *et al.* 2002; O'Brien and Campo 2002). Moreover, the down-regulation of MHC I by BPV E5 takes place not only in cultured cells but

crucially also *in vivo* in BPV-4-induced papillomas (Araibi *et al.* 2004) and this may contribute to PV escape from immunosurveillance and aid the establishment of viral infection.

The work described in this thesis extended the analysis of the functions of BPV-4 E5 and its mutants on the downregulation of MHC class I molecules in PalF cells and investigated E5 behaviour in relation to: transcription of the MHC I HC, degradation of MHC I HC protein and MHC I complex transport. This work addressed for the first time the question of whether the BPV E5 proteins downregulate MHC I through physical interaction with the complex and describes that this interaction is mediated by the C-terminus domain of E5 at least in BPV.

Moreover, other lines of evidence support the hypothesis that BPV-4 E5, as described for HPV-16 E5 (Ashrafi *et al.* 2005) is able to downregulate classical but not non-classical MHC/HLA potentially allowing the virus to evade CTL and NK killing (Araibi *et al.* 2006).

7.2 BPV-4 E5 irreversibly inhibits the transport of MHC class I molecules to the cell surface

The BPV oncoprotein E5 downregulates surface expression of MHC I, which may affect the recognition and killing of PV-infected cells by CTL (Ashrafi *et al.* 2002; O'Brien and Campo 2002). E5 interferes with several steps of the MHC I pathway, including transcription of MHC I heavy chain mRNA, expression of MHC I heavy chain protein, and transport of MHC I complex to the plasma membrane (Ashrafi *et al.* 2002).

7.2.1 Golgi apparatus retention

Both endogenous MHC I or exogenous (transfected) GFP-MHC I heavy chain fusion protein are retained in the swollen and misshapen GA characteristic of E5-expressing cells and co-localize with the E5 protein in the GA (figures 3.2 J and 3.3 A and B). The detection of endogenous MHC I in the GA with mAb IL-A19, which does not recognize free MHC I heavy chain, indicates that the heavy chain is complexed with β 2-microglobulin. This suggests that the arrest of MHC I transport occurs following MHC class I complex formation in the ER and is due instead to a fault in cellular transport across the Golgi compartments. Indeed, treatment of control cells with the ionophore monensin resulted in the acquisition of a phenotype remarkably similar to that of E5 cells, with many vacuoles and a distorted GA, with a concordant rapid and marked down-regulation of surface MHC I (figure 3.4 A, B and C). Monensin is a well-characterized inhibitor of GA acidification, which hinders cellular transport of newly synthesized proteins, particularly interfering with transfer across Golgi compartments and compromising secretion from the medial to the trans-Golgi (Tartakoff 1983). Taking into account the similar functional effect of monensin and E5, these data suggest that E5 causes GA malformation and retention of the MHC I complex in the GA at least in part by increasing GA pH.

However, the impeded transport of the MHC I complex in E5 cells is not part of a generalized disturbance of intracellular traffic as the transport of the transferrin receptor is not affected by E5 expression (Ashrafi *et al.* 2002) nor by treatment with monensin (figure 3.5 A and B). Like E5, the HTLV-1 p12^I, another small hydrophobic viral protein, binds to the 16k subunit c component of the H⁺-V-ATPase (Franchini *et al.* 1993). p12^I can also bind MHC I causing its intracytoplasmic retention and degradation (Johnson *et al.* 2001). Moreover, it has been shown that

improperly processed MHC I molecules accumulate and are degraded in an expanded Golgi intermediate compartment (Raposo *et al.* 1995). In E5-expressing cells the interaction of E5 with 16k subunit c may lead to improper processing of MHC I that then accumulates in the GA, an hypothesis that is consistent with the cellular localization of the MHC class I complex in the E5 cells.

7.2.2 BPV-4 E5 reversibly inhibits transcription of the MHC class I HC gene.

In PalF 4-E5 cells, the levels of HC RNA are barely detectable (Ashrafi *et al.* 2002), and by quantitative RT-PCR we estimate that the amount of HC RNA in these cells is between 1- and 3-fold less than in control cells (Figure 4.2 A, white bars). The reduction of HC RNA is due to a transcriptional inhibition of the HC gene (Marchetti *et al.* 2006).

In order to determine if the MHC I biosynthetic pathway was rendered irretrievably malfunctioned by E5, PalF control and E5-expressing cells were treated with type I and II IFNs which are potent inducers of MHC I expression (Agrawal and Kishore 2000). All of the cell lines tested, including the 4-E5 cells, responded to IFN treatment by increasing the level of MHC I heavy chain to approximately the same extent, although the effect was more marked with γ -IFN than with β -IFN (figure 4.2 A and B).

The downregulation of the heavy chain gene promoter is supported by two observations: E5 downregulates a BoLa HC gene promoter/enhancer (Figure 4.2 D and (Marchetti *et al.* 2006) and treatment with either β - or γ -IFN, both of which increase HC gene transcription (Agrawal and Kishore 2000), leads to accumulation of HC RNA also in PalF 4-E5 cells, and to increased activity of the BoLa HC promoter/enhancer (Figure 4.2 A, grey and black bars; 4.2 D and Marchetti *et al.*

2006). However, even in the presence of IFN, the levels of HC RNA in PalF 4-E5 cells are still approximately 2- to 3-fold lower than in control cells. This suggests that E5 expression causes a transcription inhibitor to bind to the BoLa promoter/enhancer, and that this putative inhibitor exerts its action even when IFN-induced transcription activators promote transcription. This hypothesis however requires confirmation and other explanations are possible. BPV E5 is not present in the nucleus (Burkhardt *et al.* 1989; Pennie *et al.* 1993; Zago *et al.* 2004) and its effect on transcription is therefore not direct. Rather, BPV E5 interferes with several signal transduction pathways (O'Brien *et al.* 2001; DiMaio and Mattoon 2001; Zago *et al.* 2004; Grindlay *et al.* 2005), and it is therefore likely that inhibition of the BoLa promoter/enhancer is achieved by interference with one or more of these pathways. It has already been shown that BPV-4 E5 affects the expression of cyclin A by acting indirectly on the transcriptional promoter of the cyclin A gene (O'Brien *et al.* 1999; Grindlay *et al.* 2005). BPV-4 E5 inhibition of the BoLa promoter/enhancer is another example of E5 interference with transcription regulation.

7.2.3 E5 induces the degradation of MHC class I HC

MHC class I half-life is regulated by degradation in the proteasomes and lysosomes (Bartee *et al.* 2004) and several viral proteins contribute to the destruction of MHC class I by favouring HC ubiquitination, or by inducing peptide mis-folding or complex mis-location (Hughes *et al.* 1997; Hewitt *et al.* 2002; Bartee *et al.* 2004). In PalF 4-E5 cells, levels of HC are much lower than in control cells (Ashrafi *et al.* 2002). Treatment with either proteasome or lysosome inhibitors in presence of γ -IFN rescues HC to levels comparable to those of control cells (Figure 4.3). In 4-E5 expressing cells the stability of HC is drastically reduced and the virtual absence of

HC in PalF 4-E5 cells is in agreement with the retention of MHC class I in the GA by E5 and with the degradation of mis-folded, mis-located or improperly processed MHC class I by both proteasomes and lysosomes. BPV E5 interacts with 16k subunit c of the V0 sector of the V-ATPase (Goldstein *et al.* 1991; Faccini *et al.* 1996) and this interaction results in alkalinization of the GA (Schapiro *et al.* 2000), which in turn we have argued is in part responsible for retention of MHC class I in the GA (Marchetti *et al.* 2002). The V-ATPase pump is ubiquitous in the endomembrane compartments, including the lysosomes, so it would have been expected that its inhibition by E5 would not have lead to HC degradation, or that lysosome inhibitors would have no effect on HC degradation. This apparent discrepancy may be due to different reasons. First, E5 resides mainly in the GA and may inhibit the pump only in this organelle, similar to HPV-16 E5, which alters endosomal pH but not GA pH (Disbrow *et al.* 2005); second, E5 may disable the pump only transiently (Ashby *et al.* 2001), and third, E5 interference with the pump is not complete and stronger inhibition is needed for blocking protein degradation in the lysosomes.

7.2.4 BPV-4 E5 irreversibly blocks transport of MHC class I to the cell surface

The arguments discussed above point to the fact that both E5-induced transcriptional inhibition of the HC gene and degradation of HC peptide can be overcome. However, the transport of MHC class I from the endomembranes to the cell surface is irreversibly compromised by E5. Although after treatment with IFN and proteasome/lysosome inhibitors, the amount of HC in PalF 4-E5 cells is similar to that of control cells, surprisingly the increase in surface MHC class I is negligible (Figure 4.4). This suggested additional mechanisms for retention of MHC I in the GA.

7.3 BPV-4 E5 interacts physically with HC

BPV-1 E5 interacts physically with the receptor for PDGF (Petti *et al.* 1994) and both BPV-1 E5 and BPV-4 E5 interact with 16k subunit c (Goldstein *et al.* 1991; Faccini *et al.* 1996). Given the established interaction between E5 and membrane proteins, we hypothesized that BPV-4 E5 interacts also with MHC class I HC. Indeed, this is the case and the two proteins coprecipitate not only *in vitro* (Figures 5.1 B,C and D) but also *in vivo* (Figure 5.4 A). This interaction is not an artefact as E5 is precipitated by an antibody against HC even when HC is not labelled (Figure 5.1 C, lane 5). E5 and HC interact in PalF-4 E5 cells as shown by the co-immunoprecipitation of HC with an antibody against E5 only in PalF-4 E5 cells and not in control cells (Figure 5.4 A, lanes 1 and 2). The reason for the low levels of the HC coprecipitated with 4-E5 resides is the fact that, although the levels of HC can be boosted many fold by the combined treatment of IFN and proteasome/lysosomes inhibitors, expression of E5 is not affected (Figure 4.2 C); E5 is present at barely detectable levels (O'Brien *et al.* 1999) and is therefore rate-limiting for complex formation with HC. The interaction between E5 and HC reflects a genuine relationship between the two proteins as there is agreement between interaction *in vivo* and *in vitro* and a good correlation between interaction *in vitro* and downregulation of MHC class I *in vivo*. Furthermore, interaction takes place also between HPV-16 E5 and HLA-A in human keratinocytes (Ashrafi *et al.* 2006b) and HPV-83 E5 and HLA-A2 *in vitro* (Ashrafi *et al.* 2006a), suggesting that the physical relationship between E5 proteins and HCs is a common mechanism to downregulate surface MHC/HLA class I. We do not know the MHC class I phenotype of the PalF cells but, as the *in vitro* co-immunoprecipitation experiments have been done with two different class I alleles, encoded at different loci, and E5 downregulates MHC class I in papillomas from calves of different breeds

(Araibi *et al.* 2004), it is reasonable to conclude that BPV-4 E5 can interact with and downregulate most, if not all classical class I HCs. This is indeed the case for HPV-16 E5 (Ashrafi *et al.* 2006b).

7.3.1 The interaction between BPV-4 E5 and HC is mediated by the C-terminus domain of 4-E5

4-E5T which is a C-terminus deletion mutant of BPV-4 E5 does not inhibit the expression of MHC class I or its transport to the cell surface (Ashrafi *et al.* 2002). The lack of downregulation is due to lack of interaction with HC. 4-E5T does not interact with HC (Figure 5.3 B lane 9 and C lane 3) and the interaction between 4-E5 and HC is prevented by a peptide corresponding to the C-terminus of 4-E5 (Figure 5.3 B lane 11). The C-terminus tail of 4-E5 is clearly critical to the function of the protein: 4-E5T is not transforming (O'Brien *et al.* 1999), does not induce the typical morphological changes brought about by 4-E5, and does not distort the GA (Ashrafi *et al.* 2000). The ability to bind MHC class I HC has to be added to the functions of 4-E5 C-terminus. However, the physical interaction between 4-E5 and HC appears to be necessary but not sufficient for downregulation of MHC class I as the 4-E5 mutant N17Y binds HC but does not inhibit MHC class I expression (Figures 5.3 A lane 6 and B lane 5 and 5.2 B). This situation is reminiscent of the fact that interaction between BPV-1 E5 or BPV-4 E5 and 16k subunit c is not sufficient for cell transformation (Ashrafi *et al.* 2000; Ashby *et al.* 2001) and points to a complex interaction of E5 proteins with cellular mechanisms/pathways. Nevertheless, we confirm here previous observations from this laboratory that only transformation-competent E5 proteins are capable of inhibition of MHC class I transport. It is interesting that BPV-4 E5 can potentially interact with more than one membrane

protein simultaneously. E5 interacts with 16k subunit c through the transmembrane residue 17 (glutamine in BPV-1 E5, asparagine in BPV-4 E5) (Goldstein *et al.* 1992b), and could therefore interact with HC through its C-terminus at the same time. BPV-1 E5 interacts with the PDGF receptor through glutamine 17 and the juxtamembrane aspartate 33 (Petti *et al.* 1997; Klein *et al.* 1998) in a multiprotein complex that can include 16k ductin (Goldstein *et al.* 1992a; Lai *et al.* 2000). Residue 33 is not present in either 1-E5T or 4-E5T (O'Brien *et al.* 1999), and we cannot rule out that it plays a role in the interaction with HC. However, although we have not investigated any interaction between BPV-4 E5 and PDGF receptor, cell transformation by BPV-4 E5 does not appear to depend on signalling from growth factor receptors (Zago *et al.* 2005), and it is therefore unlikely that receptor binding would compete with HC binding.

7.4 BPV-4 E5 and putative non-classical class I : possible evasion of NK killing of infected cells

As described before many pathogens have effective means of avoiding the adaptive immune response and a common feature of many viral infections is the virus-induced modulation of class I expression. However there are several examples of discrimination between classical and non-classical class I targeting by viruses. While classical class I expression is most often down-regulated by viral interference at one or more points in the pathway between transcription and cell surface expression, expression of non-classical class I genes usually remains undisturbed (Cohen *et al.* 1999; Tomasec *et al.* 2000; Wang *et al.* 2002). It has been proposed that this enables the virus-infected cell to evade cytotoxic T cell responses while at the same time sending inhibitory signals to NK cells via expression of one or more non-classical class I genes. This relates to engagement of inhibitory NK receptors by non-classical

class I molecules, for example CD94/NKG2 molecules by HLA-E, KIR2DL4 by HLA-G (Hofmeister *et al.* 2003; Borrego *et al.* 2005).

In vitro and *in vivo* immunoprecipitation experiments have shown that BPV-4 E5 binds to bovine class I HC and allowed to map the HC-interacting domain of E5 to its C-terminus end (paragraph 5.3 and (Marchetti *et al.* 2006). Recent data from this laboratory have also shown that HPV-16 E5 down-regulates classical HLA I (MHC class I) but not non-classical HLA-E (Ashrafi *et al.* 2005). To determine whether BPV-4 E5, like HPV-16 E5, is incapable of down-regulating certain non-classical MHC genes, we used a recently described gene in the bovine MHC class I cluster, *N*50001* (Di Palma *et al.* 2002). *N*50001* and other eight putative non-classical class I sequences (*N*50001-N*50501*, see <http://www.ebi.ac.uk/ipd/mhc/bola/>) have been recently identified in cattle cDNAs, and most of them have not yet been functionally characterized.

*N*50001* (GenBank accession: AY188807) was first identified in 1995 when it was termed HD59 (Ellis *et al.* 1996) and subsequently was mapped to a region of the cattle MHC containing several classical class I genes (Di Palma *et al.* 2002). *N*50001* encodes a HC with a truncated cytoplasmic domain (8 rather than 28 amino acids), and in this sense resembles human HLA-G (Geraghty *et al.* 1987) (Figure 5.5A).

Immunoprecipitation experiments showed lack of interaction between 4-E5 and *N*50001* and this prompted the question of whether the intra-cytoplasmic domain of class I HC was responsible for binding to 4-E5.

The C-terminus truncation mutant of *N*01301* HC lacking the final 19 amino acid residues still interacts with 4-E5 (figure 5.5 F lane 3). Furthermore, *N*01301* HC bound 4-E5 whether tagged with V5-His at its C-terminus, like *N*50001*, or not (figure 5.5 F lanes 11 and 7), demonstrating that the C-terminus epitope has no effect

on the interaction. These results lead to the conclusion that 4-E5 does not interact with the non-classical N*50001 MHC class I, in marked contrast to its inhibitory effect on classical MHC class I.

Moreover BPV-4 E5 does not affect either the stability of N*50001 heavy chain or the transport of N*50001 MHC I complex to the cell surface (Araibi *et al.* 2006). A major difference between classical N*01301 and non-classical N*50001 HC is the truncation of the intra-cytoplasmic domain in the latter, but the interaction with 4-E5 does not take place via this domain. This finding contrasts with the binding of HIV Nef to the intra-cytoplasmic domain of HLA-A and with the observation that the inability of Nef to bind some non-classical HLA resides in amino acid differences in the intra-cytoplasmic domain of HLA-E and the absence of the domain in HLA-G (Williams *et al.* 2002; Pizzato *et al.* 2004). There are several aminoacid differences throughout the extracellular domains between classical N*01301 and non-classical N*50001 HC (Figure 5.5A), but which one of these differences is responsible for the differential interaction with 4-E5 remains to be established.

Downregulation of classical MHC class I and lack of downregulation of non-classical MHC class I by BPV-4 E5 suggest that, like other viruses, BPV is potentially capable of evading CTL and NK killing of infected cells.

7.5 PV E5-MHC I HC interaction: a common mechanism that leads to immunoevasion ?

Despite the failure to isolate papillomavirus from equine sarcoids, there is now a large body of evidence strongly supporting the hypothesis that BPV is the etiological agent of equine sarcoids. Both BPV-1 and BPV-2 have been detected in sarcoid tumours,

with BPV-1 the predominant type (Trenfield *et al.* 1985; Angelos *et al.* 1991; Lory *et al.* 1993; Bloch *et al.* 1994; Reid *et al.* 1994).

Several studies have evaluated BPV gene expression in equine sarcoids demonstrating the presence of BPV-1 DNA, BPV specific RNA and the presence of the major BPV transforming protein E5 (Nasir and Reid 1999; Carr *et al.* 2001a; Carr *et al.* 2001b; Chambers *et al.* 2003a). These studies clearly provide evidence that the viral genes are expressed and that therefore the presence of the viral DNA is not accidental.

The role of the immune response in determining the outcome of PV infections is well established. In most cases, regression of PV lesions occurs following activation of the host immune response. However, several immune evasion mechanisms that may contribute to persistence and progression of PV-associated disease have also been described in this thesis and in (O'Brien and Campo 2002).

Sarcoid are non-regressing, in contrast to many other lesions caused by PV infection, suggesting that expression of the BPV proteins in equine cells may evoke similar immune evasion mechanisms. Results from this thesis show that 4-E5, the major oncoprotein of BPV-4, retains MHC class I molecules within the Golgi apparatus and prevents transport of MHC class I complex to the cell surface both *in vitro* and *in vivo* (figure 3.2, 5.1, 5.2, 5.4 and Marchetti *et al.* 2002; Ashrafi *et al.* 2002; Araibi *et al.* 2004; Marchetti *et al.* 2006). Moreover this impaired transport of MHC class I complex is due to the physical interaction of the MHC I HC with the C-terminus of the 4-E5 protein (figure 5.3 and Marchetti *et al.* 2006). We asked whether this type of interaction could occur between BPV-1 E5 and the equine leukocyte antigen HC. Immunoprecipitation experiments shown that 1-E5 was able to interact *in vitro* with equine HC whereas the truncated form 1-E5T that lacks the C-terminus domain was not (figures 6.2.1 D and E). In fact, like the C-terminus deletion mutant of 4-E5 (4-

E5T), 1-E5T is transformation disabled (O'Brien *et al.* 1999), and it has been shown that the down regulation of surface MHC I and cell transformation co-segregate (paragraph 5.2 and (Ashrafi *et al.* 2002; O'Brien PM and MS Campo 2003).

Moreover, the addition of 1-E5T in competition experiments did not prevent the interaction between 1-E5 and EqB2 HC or EqB4 HC (Figure 6.2.1 G) whereas the full length 1-E5 did compete with itself (Figure 6.2.1 F) indicating, conclusively, that the interaction between 1-E5 and equine HCs takes place via the C-terminus of 1-E5.

There are several amino acidic similarities throughout the C-terminus domains of BPV-1 E5 and BPV-4 E5, but which one of these aminoacid is responsible for the interaction with class I HC remains to be established.

Moreover, also HPV-16 E5 is able to interact *in vitro* with HLA-A1, -A2, -B8 and *in vivo* with HLA-A3 and the down-regulation of surface HLA I and interaction with HC are mediated by the first hydrophobic domain of HPV-16 E5 (Ashrafi *et al.* 2006b).

7.6 Role of BPV-4 E5 in papillomavirus pathogenesis

During BPV infection, despite the presence of numerous papillomas actively producing virus, early or late viral antigens are not recognised by the host immune system for a significant period of time (Chandrachud *et al.*, 1994, 1995; McGarvie *et al.*, 1995). This suggests that the host immune system is unaware of, or disabled by, BPV infection. Failure of inducing anti-viral immune responses during papillomavirus infection is believed to be due in part to the nature of the virus life cycle, which may prevent the immune cells having access to the viral proteins (Tindle 2002). As described in this thesis, BPV-4 E5 downregulates classical MHC class I at more the one level in PalF cells. However, in PalF cells, E5 was co-expressed with E6, E7 and activated *ras* (Pennie *et al.*, 1993), leaving open the possibility, albeit remote, that the

downregulation of MHC class I was due to a functional interaction between the viral and cellular oncoproteins. This is likely not the case as Araibi *et al.*, have shown that, in mouse mastocytoma P815 cells, E5 alone profoundly inhibits expression of both surface and total classical MHC class I in the absence of other viral or cellular oncoproteins (Araibi *et al.*, 2006).

It maybe argued that the results presented in this thesis are limited to the *in vitro* system. Araibi *et al.*, investigated the relationship between the expression of BPV-4 E5 and MHC class I in naturally occurring papillomas. In this study, E5 was detected exclusively in the cytoplasm of epidermal cells, from the basal and parabasal layers to the spinous and squamous layers. Cells that expressed E5 did not express MHC class I and cells that expressed BPV-4 E7, but no E5, still had detectable MHC I (Araibi *et al.*, 2004). The conclusion from this study was that although cooperation between BPV-4 E5 and E7 is necessary to achieve full transformation, downregulation of MHC I is only induced in the presence of E5, and E7 is not responsible for downregulation of MHC I.

The detection of E5 in cells from the basal to the squamous layers are in agreement with previous studies (Anderson *et al.*, 1997; Burnett *et al.*, 1992) and confirms the hypothesis of the function of E5 protein in natural infection; expression of E5 protein in the basal layer support its role in immune evasion by downregulation of MHC I in those cells that are accessible to immune surveillance. Expression of E5 protein in differentiated layers supports its role in viral replication and packaging of the viral genome (Fehrmann *et al.*, 2003). In addition, expression of E5 protein in the intermediate layers supports its role in the maintenance of the transformed state of papillomas.

7.7 Conclusions

It has been shown that BPV-4 E5-induced retention of MHC class I in the GA is linked to cell transformation (Ashrafi *et al.* 2002; O'Brien and Campo 2003) and is due to the alkalization of the GA (Marchetti *et al.* 2002). Results in this thesis suggest that direct binding between E5 and HC contributes to (but is not sufficient for) the inhibition of MHC class I transport to the cell surface. The expression of E5 is much lower than that of HC, but this stoichiometric imbalance is offset by E5-induced transcriptional inhibition of the HC gene and degradation of the HC peptide. However, E5 prevents traffic of the MHC class I complex even when the amount of HC is greatly increased by drug treatment, therefore, for E5 to exert any effect on HC, the binding must be dynamic. One possible model could be the following: E5 retains newly assembled MHC class I complex in the GA by preventing GA acidification and by physically interacting with HC; the mis-located complex is shunted to lysosomes for degradation, and E5 is free to interact with new MHC class I and re-start the process (Figure 7.6). While aspects of this model need confirming, the absence of MHC class I in the E5-expressing cells of naturally occurring BPV-4 induced papillomas supports our suggestion that E5 plays an important role in the establishment and persistence of viral infection by allowing the infected cells to escape host immunosurveillance (Araibi *et al.* 2004).

Other viral proteins, such as HIV Nef (Williams *et al.* 2002) and HCMV US2 and US8 (Chevalier *et al.* 2002 ; Tirabassi *et al.* 2002), to name but a few, bind HC and downregulate surface MHC class I, and E5 now joins this large number. It is interesting to point out the parallels between E5 and Nef. Nef binds both Vma13, a component of the V1 sector of the vacuolar ATPase, facilitating the internalization of surface receptors (including MHC class I), and MHC class I HC, disrupting MHC

class I traffic (Williams *et al.* 2005; Lu *et al.* 1998). Furthermore, as for E5, the same domain of Nef necessary for disruption of MHC class I traffic, mediates the binding to the HC (Williams *et al.* 2005).

BPV-4 E5 interacts with two different alleles of classical MHC class I HC (N*01301 and N*00201) *in vitro* and the interaction has been confirmed *in vivo* in PalF cells, BPV-1 E5 interacts with two different equine classical class I HC (EqB2 and EqB4) *in vitro*, the HPV-16 E5 interacts with classical HLA-A3 in HaCaT cells, and A1, A2 and B8 *in vitro* (Ashrafi *et al.* 2006b) and HPV-83 E5 interacts with HLA-A2 (Ashrafi *et al.* 2006a). These results show that all the E5 proteins tested, independent of the PV type, are able to interact with different classical class I alleles.

These observation add a new step in the general downregulation of MHC class I by E5 proteins and point to a common pathway of immunevasion by E5s from different PV types.

This similarity is intriguing and supports the hypothesis that the carcinogenicity of papillomavirus types reflects viral evolution with carcinogenic PV types (HPV-16, BPV-1, BPV-4) coding for an E5 protein, whereas most low-risk types either lack a definable homologous E5 ORF and/or a translation start codon for E5 (Schiffman *et al.* 2005). Moreover, in a study on mucosal HPVs Bravo and Alonso have shown that E5-like proteins can be classified into four groups according to their chemical characteristic and evolutionary relationships. This classification matches the epidemiological characteristics of the mucosal HPVs and their differential association with cancer development (Bravo *et al.* 2004; Clifford *et al.* 2003; Munoz *et al.* 2003). Like HPV-16 E5, BPV-4 E5 is incapable of down-regulating certain non-classical MHC genes (Ashrafi *et al.* 2005, paragraph 5.5 and Araibi *et al.* 2006). These results suggest that BPV-4 E5 could potentially provide a mechanism for the escape of the

infected cells from not only CTL but also NK killing as it has been shown for other viral proteins like HIV Nef and HCMV UL40 (Cohen *et al.* 1999; Tomasec *et al.* 2000; Ulbrecht *et al.* 2000).

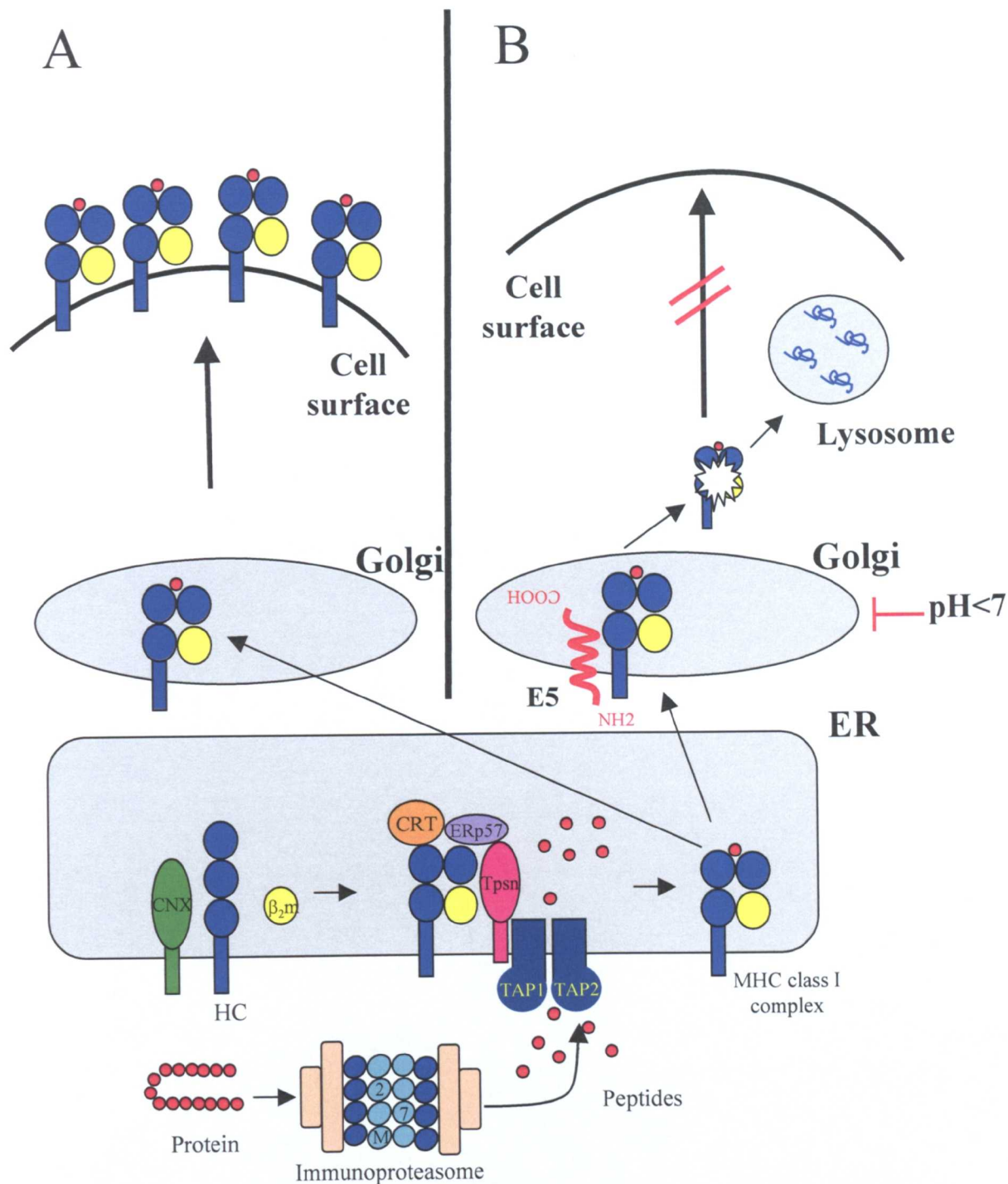


Figure 7.6 Possible model of MHC class I trafficking in BPV E5-expressing cells (B) compared with the normal MHC class I trafficking pathway (A). After the assembly and peptide loading in the ER (discussed in detail in section 1.1.3.1 and fig. 1.4), MHC I molecules transit through the Golgi apparatus before reaching the cell surface for presentation to CTL cells. (B) In BPV E5-expressing cells E5 retains newly assebled MHC class I complex in the Golgi apparatus (GA) by preventing GA acidification and by physically interacting with HC; the mis-located complex is directed to lysosomes for degradation and E5 is free to interact with new MHC class I and re-start the process.(Picture adapted from Van Kaer ,*Tissue Antigens* 2002). HC, heavy chain; CNX, calnexin; β_2m , β_2 -microglobulin; CRT,calreticulin; Tpsn, tapasin; 2, LMP2; 7,LMP7; M, MCL-1.

7.8 Future work

It has been shown that the BPV-4 E5 C-terminus domain is involved in the interaction with the MHC I heavy chain (HC). Further studies are needed to establish which amino acid(s) within the C-terminus domain is/are essential for this type of interaction.

Further analysis on which domain of MHC I heavy chain is involved in this interaction are required. It has been shown that the HC C-terminus domain is not involved but further mutation analysis of heavy chain will identify which amino acid(s) is/are responsible for the interaction.

Lack of surface MHC I and the presence of non-classical MHC I in infected cells expressing E5 would allow evasion of cytotoxic T lymphocyte and NK killing and thus establishment of viral infection. CTL and NK killing assays on E5-expressing cells would clarify this point.

Bovine papillomavirus has been used as model system in which to study the interaction of a papillomavirus with its natural host and with environmental cofactors (Jarrett *et al.* 1978; Campo *et al.* 1994b) and has been important in the recognition of the oncogenic nature of the virus and the development of anti-viral vaccine (Campo 1997). Comparative pathology studies between BPV and HPV-induced diseases have contributed to the understanding of HPV-induced cancer in humans. Moreover the latest insights in BPV E5, E2 (Zhao *et al.* 2000; Abroi *et al.* 2004) and E7 (Narechania *et al.* 2004) functions show that BPV can still lead the way and contribute significantly to our understanding of virus biology.

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Appendix (a)

Down-regulation of MHC class I by bovine papillomavirus E5 oncoproteins

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The papillomavirus E5 protein is localized in the endoplasmic reticulum (ER) and Golgi apparatus (GA) of the host cell. Transformed bovine fibroblasts expressing bovine papillomavirus (BPV) E5 are highly vacuolated and have a much enlarged, distorted and fragmented GA. Major histocompatibility complex class I (MHC I) is processed and transported to the cell surface through the GA. Given the cellular localization of E5 in the GA and the morphologically abnormal GA, we investigated the expression of MHC I in cells transformed by E5 from BPV-1 and BPV-4. Two cell lines were used: bovine cells that also express E6, E7 and activated ras, and NIH3T3 cells that express only E5. In addition, PalF cells acutely infected with a recombinant retrovirus expressing E5 were also examined. In contrast to non-transformed normal cells, or transformed cells expressing other papillomavirus proteins, cells expressing E5 do not express MHC I on their surface, but retain it intracellularly, independently of the presence of other viral or cellular oncogenes, or of whether the cells are long-term transformants or acutely infected. We conclude that expression of E5 prevents expression of MHC I to the cell surface and causes its retention within the cell. In addition, lower amounts of total MHC I heavy chain and of heavy chain RNA are detected in E5-transformed cells than in control cells. As surface expression of another glycosylated membrane protein, the transferrin receptor, is not affected, it appears that E5 targets MHC I with at least a degree of specificity. In papillomavirus lesions this effect would have important implications for antigen presentation by, and immunosurveillance of, virally infected cells.

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Keywords: papillomavirus; E5; MHC class I; Golgi apparatus; cell transformation

Introduction

Papillomavirus is an oncogenic virus which infects mucosal and cutaneous epithelia where it induces benign hyperproliferative lesions. In the great majority of cases, papillomavirus infections are usually cleared, after a variable period of time, usually several months, by a cell mediated immune response directed against viral antigens (Frazer and Tindle, 1996). Occasionally however, the lesions do not regress and can progress to cancer. Persistent viral infection is required for neoplastic progression and failure of virus clearance is attributed to a poor immunological response.

The papillomavirus genome encodes three proteins, E5, E6 and E7, capable of inducing unscheduled proliferation of keratinocytes. All three proteins can transform cells *in vitro*, although their contribution to cell transformation appears to vary in different virus types. While E6 and E7 are the main oncoproteins of mucosal HPVs (Kubbutat and Vousden, 1996; Kunhe and Banks, 1999), E5 is the major oncoprotein of BPVs, particularly of BPV-1. The E5 protein, and the closely related E8 protein of subgroup B bovine papillomaviruses (BPV), therein collectively designated E5 (Morgan and Campo, 2000), is a small hydrophobic peptide (from 83 amino acid residues in human papillomavirus type 16 [HPV-16] to 42 residues in BPV-4) which is expressed in the deep layers of the infected epithelium (Anderson *et al.*, 1997; Burnett *et al.*, 1992; Chang *et al.*, 2001). While E6 and E7 are present throughout the course of the disease and their functions are necessary for the maintenance of a transformed state, expression of E5 takes place early in infection, and is often, but not always (Chang *et al.*, 2001), extinguished in frank cancers. In genital lesions induced by mucosal HPV, the expression of E5 is extinguished as the lesion progresses to malignancy, due to the frequent occurrence of the integration of the viral genome into the host chromosome at the E2/E5 open reading frame (ORF); in the lesions induced by BPV the mechanisms that silence the expression of E5 from an intact episomal genome are not known.

E5 is localized in the endomembrane compartments of the endoplasmic reticulum (ER) and Golgi apparatus (GA) of the host cell (Burkhardt *et al.*, 1989; Pennie *et al.*, 1993). BPV-4 E5 induces anchorage independence (Pennie *et al.*, 1993), allows cell growth in low serum,

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prevents contact inhibition (Ashrafi *et al.*, 2000; O'Brien *et al.*, 1999) and down-regulates gap junction communication (Ashrafi *et al.*, 2000; Faccini *et al.*, 1996). The ability of the E5 expressing cells to grow independent of substrate and in low serum is accompanied and likely due to activation of cyclin A-cdk2 (O'Brien and Campo, 1998), while the down-regulation of gap junction communication, observed also in cells expressing HPV-16 E5 (Oelze *et al.*, 1995), is accompanied by binding of E5 to the 16 k ductin/subunit c, a component of the gap junction and of the V0 sector of the vacuolar H⁺-ATPase (Finbow *et al.*, 1991; Goldstein *et al.*, 1991; Conrad *et al.*, 1993; Faccini *et al.*, 1996). This physical interaction has been deemed responsible also for the lack of acidification of endosomes and GA (Schapiro *et al.*, 2000; Straight *et al.*, 1995), which has been attributed to a malfunction of the H⁺-ATPase (Briggs *et al.*, 2001), although inhibition of the vacuolar proton pump is not always observed (Ashby *et al.*, 2001). Cellular transformation by E5 is also accompanied by the activation of growth factor receptors (Martin *et al.*, 1989; Petti *et al.*, 1991; Leechanachai *et al.*, 1992; Straight *et al.*, 1993) and other kinases (Gu and Matlashewski, 1995; Crusius *et al.*, 1999; Supryniewicz *et al.*, 2000). It is reasonable to assume that the various aspects of cell transformation by such a small protein may all derive from its very location in the endomembrane compartment, particularly the GA, with the putative consequent disruption of exocytic and endocytic cellular traffic, including transport of the MHC I complex.

Recognition and elimination of virally infected cells by T-lymphocytes require presentation of viral peptides by the MHC class I complex. Peptides are loaded onto the MHC I heavy chain in the ER, where β 2-microglobulin and chaperones, such as TAP, associate with the complex. MHC I is glycosylated as the complex is transported from the ER through the GA to the plasma membrane for recognition by T-cells (Cresswell *et al.*, 1999). Deglycosylation in the GA is necessary for the dissociation of MHC I from both calreticulin and TAP peptide transport molecules (Van Leeuwen and Kears, 1996). In addition, the assembly of the MHC I complex with peptide is pH dependent (Reich *et al.*, 1997). Therefore, given the cellular location of E5 in cell membranes and the attendant inhibition of membrane compartment acidification, we investigated whether MHC I would be properly transported to the cell surface. We report that in E5-expressing cells MHC I is down-regulated and retained intracellularly, thus adding yet another function to the pleiotropic action of this protein.

Results

Morphology of Golgi apparatus in PalF cells

As reported previously (Ashrafi *et al.*, 2000), the E5-expressing transformed PalF cells were very enlarged and highly vacuolated (Figure 1a). Vacuole formation takes place only in the presence of the full transform-

ing forms of E5. The vacuoles are compartmentalized, with vacuoles communicating within but not between compartments as shown by the distribution of the fluorescent dye lucifer yellow injected into single cells (Ashrafi *et al.*, 2000).

E5 inhibits acidification of the endomembrane compartments, and it is known that the inhibition of acidification of endomembrane compartments by ionophores leads to fragmentation of the Golgi apparatus. Therefore, to investigate the origin of the vacuoles in E5 cells, we visualized the GA by immunofluorescence. Monoclonal antibody (mAb) 4A3 is raised against GM130, an integral component protein of Golgi membranes (Barr *et al.*, 1998). When PalF cells or transformed control cells were incubated with mAb 4A3 the GA showed a normal morphology (Figure 1b). In the E5-expressing cells, the GA showed a disrupted architecture and numerous vacuoles had reacted with the antibody (Figure 1c). Often only vacuoles were stained (Figure 1c, left panel). Similar

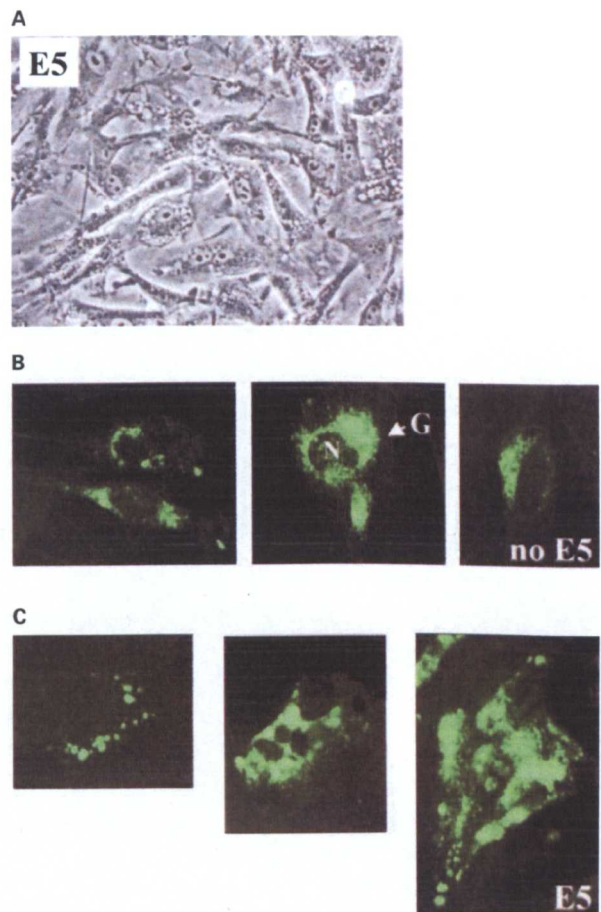


Figure 1 Golgi apparatus morphology in parental and transformed PalF cells. Cells were stained with mAb 4A3. (a) Phase contrast field of highly vacuolated 4-E5 expressing cells. (b) Examples of transformed control cells (no E5); (c) Examples of 4-E5 transformed cells. (G) Golgi apparatus (indicated by an arrow in one of the b panels); N: nucleus. Magnification: $\times 10$ in (a); $\times 40$ in all the other panels

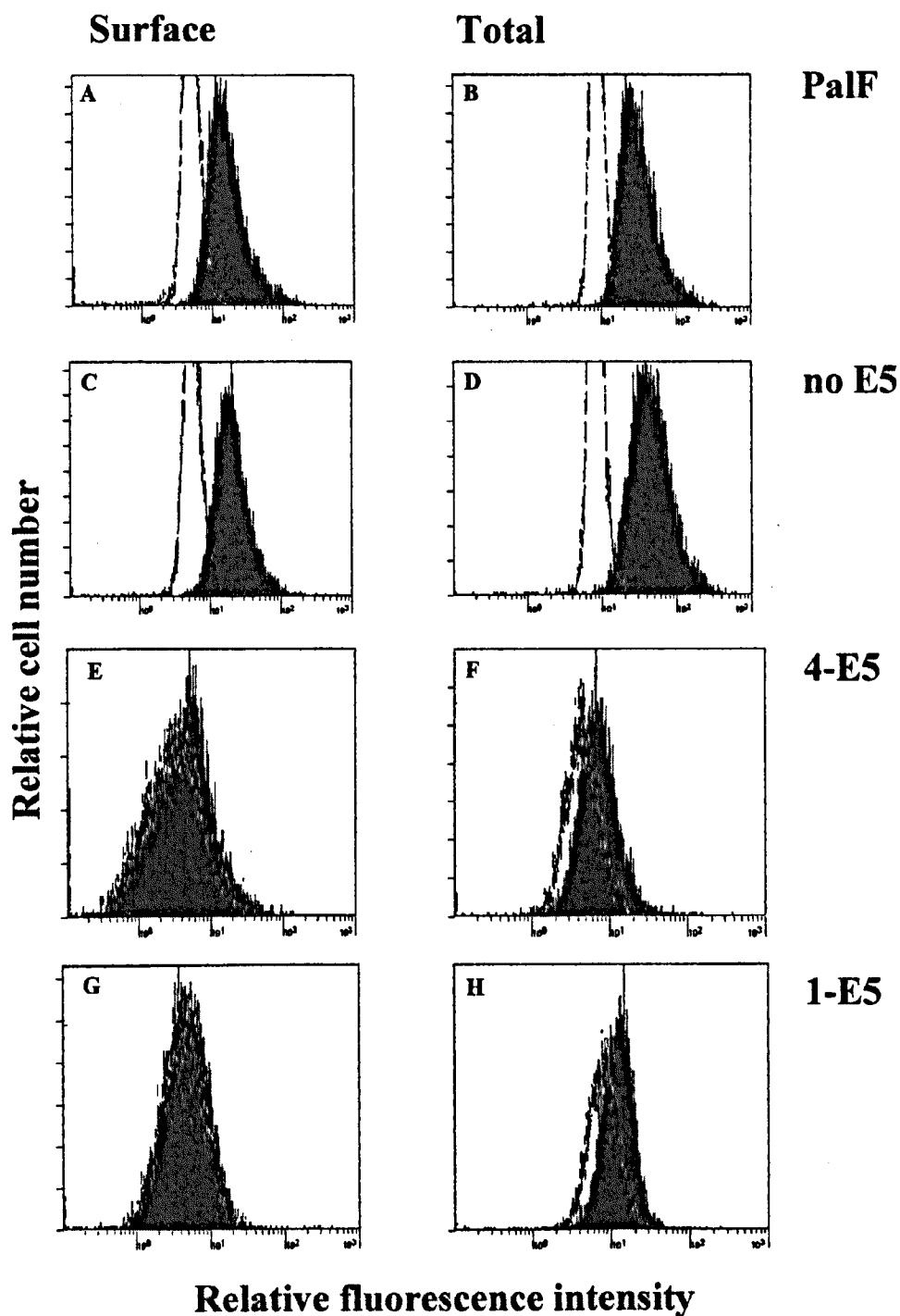


Figure 2 FACS profiles of MHC I expression in parental and transformed PalF cells. PalF cells (a,b), transformed control cells (c,d), 4-E5 transformed cells (e,f) and 1-E5 transformed cells (g,h) were stained with mAb IL-A19 and analysed by flow cytometry. Surface MHC I was measured in intact cells (a,c,e,g) and total MHC I was measured in saponin-permeabilized cells (b,d,f,h)

results were obtained when the GA was stained with fluorescent BODIPY-ceramide, which localizes to the Golgi membranes, or when it was visualized by electronmicroscopy (data not shown). These results suggest that at least some of the vacuoles in E5 cells are derived from the fragmentation of the Golgi

cisternae. The fragmentation of the GA in E5 cells does not appear to be a consequence of long-term culturing as in approximately one-third of PalF cells acutely infected with a recombinant retrovirus expressing BPV-4 E5 (see below) the GA was enlarged, when compared with cells infected with the control retro-

virus, although not as distorted as in long-term E5 transformants (data not shown).

Morphology of Golgi apparatus in NIH3T3 cells

To ascertain the state of the GA in cells other than PalF cells, we investigated the morphology of the GA in NIH3T3 cells expressing BPV-4 E5 using mAb 4A3. Despite the alkaline pH of the GA in the 3T3 E5 cells (R Schlegel and S Campo, unpublished observations), there were no obvious structural abnormalities between the GA in control NIH3T3 and that detected in the E5 cells (data not shown). This suggests that the abnormal GA morphology observed in PalF cells expressing E5 may be cell type dependent.

MHC class I on the cell surface

Given the abnormal morphology of the ER and GA in E5-expressing PalF cells, and given the importance of these structures in protein traffic, we investigated whether E5 expression would disrupt protein transport and in particular MHC I transport to the cell surface. Parental PalF and transformed control cells were incubated with IL-A19, a monoclonal antibody recognizing a monomorphic determinant of the bovine MHC class I complex (Bensaid *et al.*, 1989) and analysed by flow cytometry. Both cell lines displayed MHC I on their surface as shown by the increase in relative fluorescence intensity in the presence of mAb IL-A19 (Figure 2a,c). In contrast, there was no shift in fluorescence in the transformed cells expressing E5 (Figure 2e,g) indicating that these cells do not present MHC I on their surface. To investigate if this was due to a defect in MHC I transport to the cell surface, the cells were permeabilized with saponin before staining to allow the penetration of the antibody inside the cells and therefore measuring the total MHC I content. The permeabilized parental and transformed control cells exhibited a similar staining profile, not different from that obtained for surface MHC I (Figure 2b,d). In contrast, the FACS profiles for the permeabilized E5 cells showed a positive staining for MHC I (Figure 2f,h), indicating that in these cells MHC I was retained intracellularly and was not transported to the plasma membrane. However, the intensity of fluorescence and the mean fluorescence of the E5 permeabilized cells were also noticeably reduced when compared to the parental or transformed control cells (Figures 2f,h and 7a), indicative of reduced levels of total MHC I in E5 cells.

Levels of total MHC class I

The FACS analyses above suggested a reduced level not only of surface but also of total MHC I in E5 transformed cells. To confirm this observation, the amount of total MHC I in the control and transformed cell lines was investigated by immunoblotting using mAb IL-A88, specific for a monomorphic determinant on the bovine MHC I heavy chain (Toye *et al.*, 1990).

While the MHC I heavy chain was easily detectable in PalF and control cells, little MHC I could be detected in the 1-E5 or 4-E5 cells when high amounts of protein were used (Figure 3a), confirming that E5 down-regulates expression of MHC I. The difference in sensitivity of detection of total MHC I in E5-expressing cells by FACS or immunoblotting is likely to be due to the different affinities of IL-A19 and IL-A88 antibodies for the bovine MHC I antigen (data not shown).

Levels of MHC class I transcripts

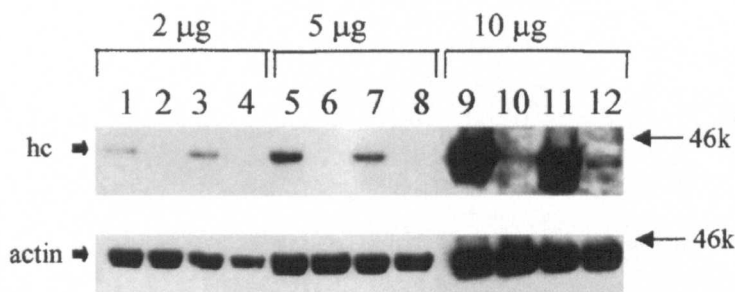
The noticeable reduction in the levels of MHC I heavy chain could be due to protein degradation, transcription inhibition, rapid RNA degradation or combinations thereof. To ascertain this point, we analysed the level of transcripts encoding the heavy chain of the MHC I complex by semi-quantitative RT-PCR, using generic oligonucleotide primers that amplify a 733 base-pair (bp) fragment from all classical bovine MHC class I genes and a 533 bp fragment from the corresponding mRNA (Sawhney *et al.*, 1995). As a control we amplified actin cDNA using primers specific for bovine actin (Sibbet *et al.*, 2000). Amplifications were carried out for progressively increasing numbers of cycles. While the 273 bp fragment of actin RNA was amplified from both non transformed PalF and 1-E5 transformed cells with similar kinetics (visible on gel after 20 cycles, Figure 3b), the amplification kinetics of MHC I heavy chain RNA were different in PalF and E5-transformed cells. In PalF cells, the expected 533 bp fragment was visible after 20 cycles; in contrast in both 1-E5 and 4-E5 transformed cells the amplicon could not be detected until 25 cycles and in lower amounts (Figure 3b). A 733 bp fragment was not detected either in the test reactions or in the control reactions (with no RNA), confirming that the amplification did not involve genomic DNA. This result shows that the observed down-regulation of MHC I heavy chain protein is not due solely to protein degradation but also to transcription inhibition or RNA degradation.

MHC I on the cell surface in transformed NIH3T3 cells

The transformed PalF cells analysed above express a variety of oncogenes and it could be argued that down-regulation of MHC I is due to the interaction of E5 with the E6, E7 or ras oncoproteins. Therefore NIH 3T3 cells expressing only BPV E5 were also analysed for the expression of MHC I. Control 3T3 cells expressed similar levels of surface and total MHC I (Figures 4a,b and 7b). However, 3T3 4-E5 cells, like the transformed E5 PalF cells, did not express MHC I on the surface but retained it intracellularly (Figures 4c,d and 7b). These results show that inhibition of MHC I transport is due to the expression of the E5 protein *per se* and not to its interaction with other oncoproteins.

However in contrast to the transformed PalF cells, the E5-transformed 3T3 cells did not show any noticeable reduction in total MHC I. The reduction

A



B

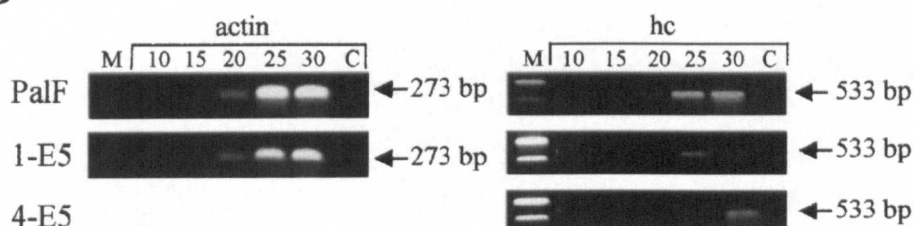


Figure 3 (a) Western blot analysis of MHC I heavy chain expression in protein lysates from parental and transformed PalF cells. Top panel, MHC I heavy chain detected with mAb IL-A88. Bottom panel, actin detected with mAb AB-1. Amount of protein loaded is indicated. Lanes 1,5,9: parental PalF, lanes 2,6,10: 1-E5 transformed cells; lanes 3,7,11: control transformed cells; lanes 4,8,12: 4-E5 transformed cells. The secondary antibody was used at a dilution of 1:5000 in lanes 1–8 and 1:2000 in lanes 9–12. The arrows on the right point to the 46 kDa marker and the bold arrows on the left to the MHC I heavy chain (hc) and actin. (b) Semi-quantitative RT-PCR analysis of MHC I heavy chain RNA in parental and transformed PalF cells. Amplicons were generated in increasing cycles of amplification from 10 to 30, as indicated. Left panels, actin RNA from PalF and 1-E5 cells. Right panels, MHC I Heavy chain RNA from PalF and E5 transformed cells. Lane c: amplification reactions with no RNA; M: the 500 and 600 bp bands of the DNA ladder marker

of total MHC I in PalF cells may be due therefore to the combined effects of the viral oncoproteins and/or activated ras, the enlargement and fragmentation of the GA, and to transcriptional down-regulation.

MHC I on the cell surface in PalF cells acutely infected with recombinant retroviruses

The PalF and 3T3 transformed cell lines above are established lines and their long-term culture may have affected the down-regulation of surface MHC I. Hence, PalF cells acutely infected with the recombinant 4-E5 retrovirus were analysed for MHC I expression. Again, these cells showed a marked reduction in surface MHC I (Figure 5c) and a less pronounced reduction in total MHC I (Figure 5d), remarkably similar to that observed in the established transformed cells (Figure 7a). This reduction was not observed in PalF cells infected with the control retrovirus (Figure 5a,b), confirming that E5 is indeed the cause of the inhibition of MHC I expression.

Transferrin receptor

To ascertain whether the down-regulation of surface MHC I by E5 was specific or due to a generalized disturbance in exo- or endocytic protein transport, we

analysed cell surface presentation of another membrane glycoprotein, the transferrin receptor. There was no difference between the total or cell surface amount of transferrin receptor in parental PalF, control or E5 cells (Figure 6 and data not shown). Therefore synthesis and maturation of glycoproteins is not generally affected in E5 transformed cells, indicating at least a degree of specificity for the E5 down-regulation of MHC I.

Down-regulation of surface MHC I and cell transformation co-segregate

A panel of PalF cell lines expressing mutant forms of the E5 protein (Ashrafi et al., 2000) was analysed for down-regulation of surface MHC I. The E5 mutants used here were an hyper-transforming form of BPV-4 E5 in which an asparagine residue at position 17 has been substituted with alanine (N17A); two mutant E5 forms that have completely lost their transformation ability, N17Y, in which residue 17 has been substituted with tyrosine, and the C-terminal truncation mutant E5T, which has no hydrophilic tail, and BPV-1 E5T, a similar C-terminal truncation mutant. Only the hyper-transforming mutant of 4-E5 was capable of inhibiting MHC I transport to the cell surface (Figure 7c). There was no difference between total and surface MHC I in

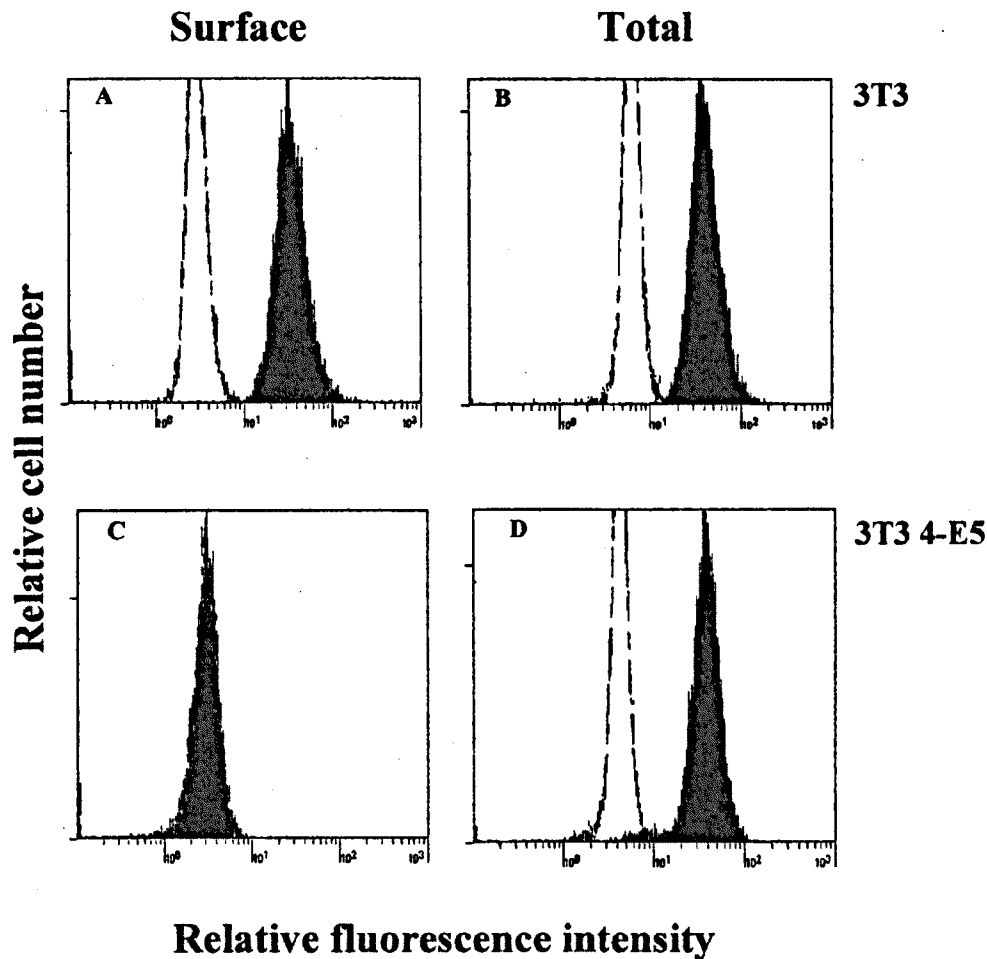


Figure 4 FACS analysis of MHC I expression in NIH 3T3 and NIH 3T3 E5 cells. NIH 3T3 cells carrying empty vector (a,b), or expressing 4-E5 (c,d) were stained with mAb CL9011-A and analysed by flow cytometry. Surface MHC I was measured in intact cells (left panels a,c) and total MHC I was measured in saponin-permeabilized cells (right panels b,d)

cells expressing transformation defective E5 mutants, although a slight decrease in surface MHC I was observed in cells expressing BPV-1 E5T (Figure 7c).

Discussion

During viral infection or malignant transformation, a spectrum of peptides derived intracellularly from viral or transformation-associated antigens is bound to MHC I molecules and displayed on the surface of the infected or tumour cell. These class I/peptide complexes are recognised by specific cytotoxic T lymphocytes (CTL), which kill the infected or malignant cells. MHC I molecules therefore play a crucial role in immune recognition and clearance of these types of cell.

Viruses need to escape immunosurveillance by the host to establish infection and to generate new infectious progeny. It is well known that viruses have developed a number of different mechanisms to avoid immune recognition by CTL by interfering with

various steps of the MHC I pathway, from prevention of peptide processing to formation of non functional MHC I complexes (see Yewdell and Bennink, 1999 for review). In each of these strategies, the outcome is the failure by the infected cell to present viral peptide antigens to cells of the immune system.

Papillomaviruses are poorly immunogenic. The prolonged absence of an immune response even in immunocompetent hosts has been attributed to a variety of causes (reviewed by Frazer *et al.*, 1999). The viral proteins are expressed in the suprabasal layers of the epithelium, i.e. in cells not readily exposed to the immune system, and moreover are produced in very low amounts, often below the threshold required for the activation of immune cells, thus leading to tolerance. We report here a possible new mechanism of immune evasion of papillomavirus: the down-regulation of MHC I by the early E5 oncoprotein.

BPV E5 is localized in the endomembrane compartment of the cell. Expression of the protein in primary bovine cells leads to profound changes in cell morphology, with extensive vacuolization (Ashrafi

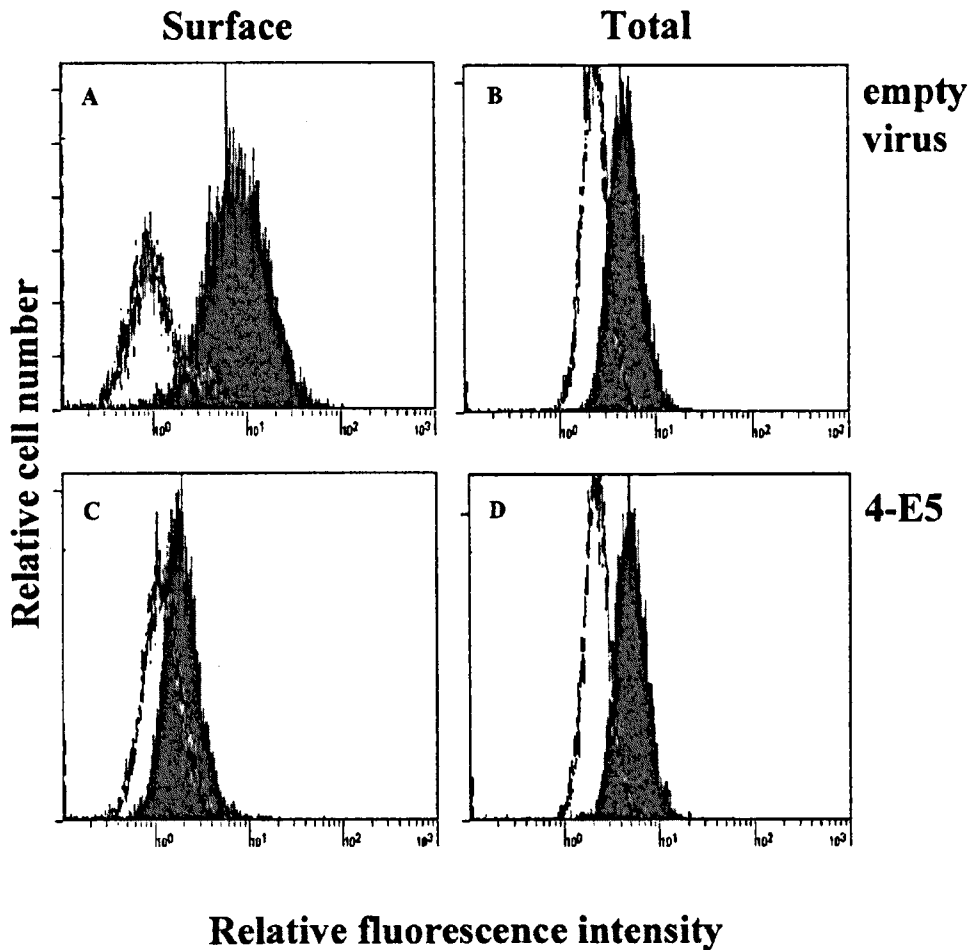


Figure 5 FACS analysis of MHC I expression in PalF cells acutely infected with E5-recombinant retrovirus. PalF cells infected with empty retrovirus (a,b) or the 4-E5 retrovirus (c,d) were stained with mAb IL-A19 and analysed by flow cytometry. Surface MHC I was measured in intact cells (left panels a,c) and total MHC I was measured in saponin-permeabilized cells (right panels b,d)

et al., 2000); the GA is particularly affected being grossly distorted, swollen and fragmented. Transformed cells that do not express E5 do not present any apparent GA malformation, so we conclude that it is the presence of the small hydrophobic E5 protein in the GA itself that induces these changes. This conclusion is supported by the swelling of the GA in cells acutely infected with an E5-expressing recombinant retrovirus, a result that also suggests that GA alterations do not depend on the interaction of E5 with the other oncoproteins present in long-term transformants. Interestingly, mutant forms of E5 which have lost or maintain only residual transformation ability do not cause noticeable changes in cell morphology or vacuolisation (Ashrafi *et al.*, 2000). However, gross disruption of the GA does not occur in NIH3T3 cells that express only E5, despite their impaired GA acidification (R Schlegel and S Campo, unpublished observation), so perhaps the morphological alteration of the GA observed in primary bovine cells, the virus natural host, is cell type-dependent.

Proper functioning of the Golgi apparatus is necessary for a number of cellular processes including protein transport to the cell surface. MHC I is synthesized in the ER, post-translationally modified in the GA and transported to the plasma membrane (Cresswell *et al.*, 1999). This process appears to be inhibited in cells expressing E5, which have very little, if any, MHC I on their surface. Down-regulation of surface MHC I takes place both when E5 is expressed with other viral transforming proteins or alone, in long-term transformants or in acutely infected cells, and whether or not the GA has undergone distortion and fragmentation. Therefore, the down-regulation of surface MHC I is due solely to E5 and does not require either other papillomavirus proteins or activated Ras. Moreover, MHC I down-regulation is not confined to BPV E5 proteins, as preliminary experiments show that NIH 3T3 cells expressing either HPV-6 or HPV-16 E5 have lower levels of surface MHC I, as do PalF cells acutely infected with a retrovirus expressing HPV-16 E5 (data not shown). Therefore, inhibition of surface MHC I expression appears to be a common character-

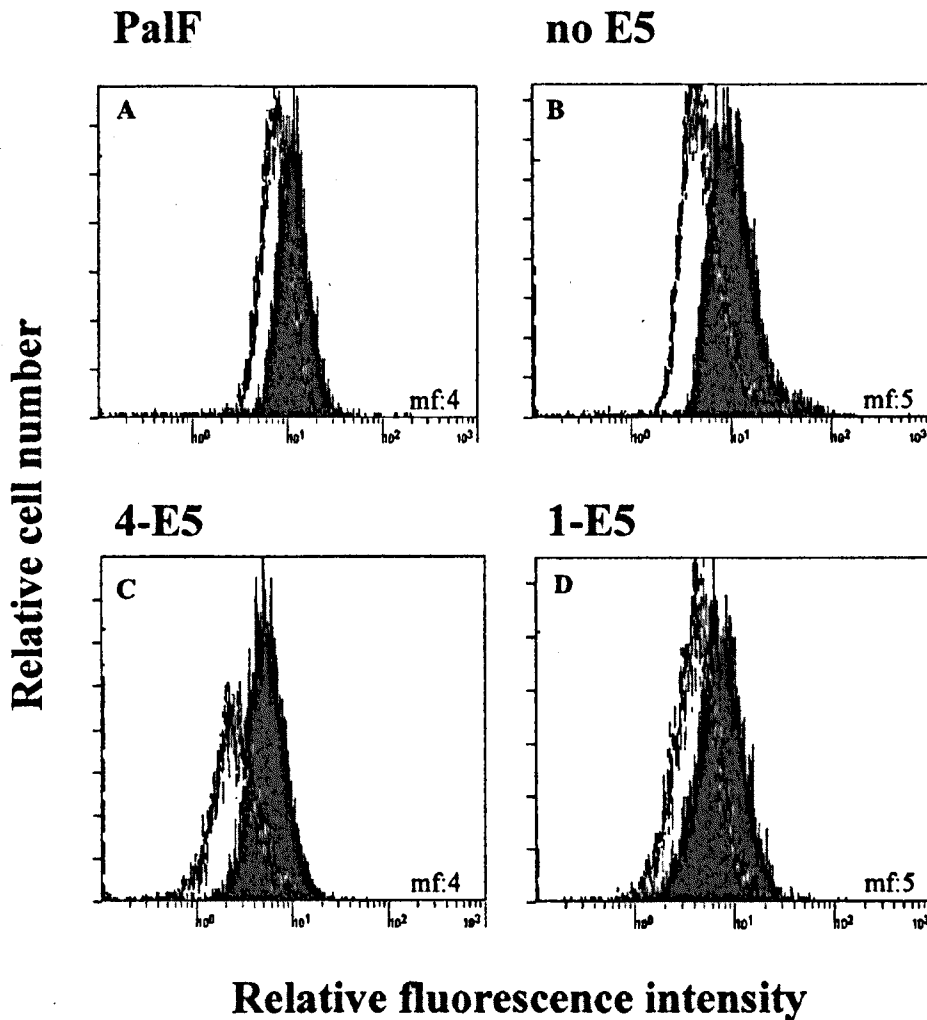


Figure 6 FACS analysis of surface transferrin receptor expression in parental and transformed PalF cells. Intact cells were stained with mAb IL-A165 and analysed by flow cytometry. The mean fluorescence (mf) values for the individual cell lines, calculated from the flow cytometric analyses, are inside the respective panels

istic of both HPV and BPV E5 oncoproteins. In addition, there is at least a degree of specificity in the disturbance of protein traffic by E5 as the expression at the cell surface, and presumably the transport, of the transferrin receptor, another membrane glycoprotein, is not affected.

The absence of MHC I on the cell surface appears to be due in part to intracellular retention as MHC I is detected once the cells are permeabilized. There is however, less total MHC I in PalF cells that express E5 than in control cells. We also detected lower levels of MHC I mRNA transcripts in the E5 cells. It is not possible at this stage to ascertain the contribution of transcription inhibition and of protein degradation to the overall down-regulation of MHC I, but it would appear that the reduced levels of MHC I heavy chain protein cannot be solely due to lack of RNA, and it is likely that both processes play a part. However, inhibition of transcription is directly due to E5 expression: the presence of approximately equal

amounts of MHC I in parental cells and transformed control cells (expressing E7) argues against transcriptional down-regulation of the heavy chain gene by E7, as recently suggested (Georgopoulos *et al.*, 2000). Moreover, expression of MHC I in HPV-positive cervical carcinomas, expressing E7 but not E5, has also been shown to be post-transcriptionally controlled (Cromme *et al.*, 1993).

The reason why the level of total MHC I remains unaltered in E5-expressing NIH3T3 cells remains to be investigated, but it may be linked to the unaltered morphology of the GA in these cells. MHC I may be degraded more rapidly in the grossly distorted GA compartment of PalF cells. This hypothesis is supported by the finding that improperly processed MHC I molecules accumulate and are degraded in an expanded ER-Golgi intermediate compartment (Raposo *et al.*, 1995).

We have not yet determined the mechanism of how E5 expression results in transcriptional inhibition and

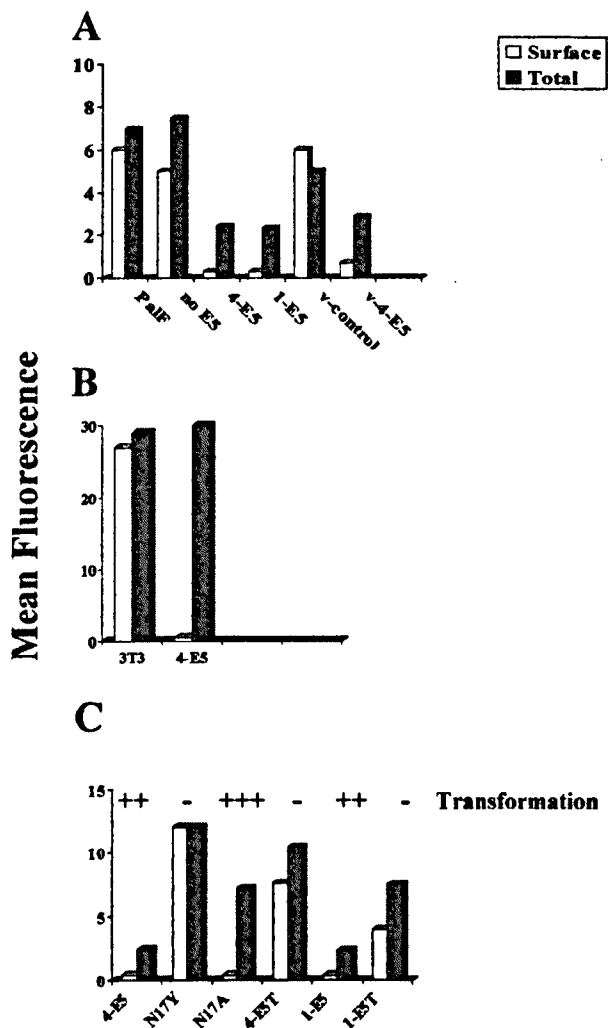


Figure 7 Representative mean fluorescence for surface or total MHC I in transformed cells. The mean fluorescence was calculated from the flow cytometric analyses above. (a) Surface and total MHC I mean fluorescence in control (PalF, no E5), transformed cells expressing BPV-4 E5 (4-E5) or BPV-1 E5 (1-E5), PalF cells acutely infected with empty retrovirus (v-control) or retrovirus expressing 4-E5 (v-4-E5). (b) Surface and total MHC mean fluorescence in NIH 3T3 cells carrying empty vector (3T3) or expressing BPV-4 E5 (4-E5). (c) Surface and total MHC I in transformed PalF cells expressing mutant forms of 4-E5 (N17Y, N17A, 4-E5T) and of 1-E5 (1-E5T). The plus and minus signs above the histogram refer to the degree of cell transformation (for more details concerning the cell lines expressing mutant E5 proteins see Ashrafi *et al.*, 2000)

loss of surface MHC I. We have shown previously that expression of E5 leads to changes in transcriptional control (O'Brien and Campo, 1998; O'Brien *et al.*, 1999), although the mechanisms used by this membrane-localized protein to signal to the transcription machinery in the nucleus are not known. The MHC I transport pathway may be blocked in the journey of the peptide/MHC complex to the cell surface, or the MHC complex could be rapidly internalized once it has reached the cell surface. The recognition of MHC I by mAb IL-A19, which detects MHC I heavy chain

complexed with β -2 microglobulin (Bensaid *et al.*, 1989), suggests that the block is a relatively late event in the genesis and transport of MHC I. Therefore, we would expect that inhibition of TAP function (Cromme *et al.*, 1994; Keating *et al.*, 1995) which is responsible for loss of MHC I in pre-malignant and malignant cervical disease (Bontkes *et al.*, 1998; Brady *et al.*, 2000), and in HPV-associated laryngeal papillomas (Vambutas *et al.*, 2000), is unlikely to play a role in E5-induced MHC I loss. This is more likely to be a feature of malignancy as observed for other malignant tumours (Seliger *et al.*, 1997). However, this point remains to be investigated experimentally. Interestingly, HPV-16 E5 has been shown recently to inhibit endocytic trafficking (Thomsen *et al.*, 2000), a finding supporting the blocking of MHC I following complex assembly.

There have been other examples of viral proteins with similar functions to E5 interfering with MHC I. It has been shown that HIV-1 Nef binds Vma13, one of the components of the hydrophilic VI sector of the V-ATPase (Lu *et al.*, 1998) and causes the accumulation of endocytosed MHC I in the *trans*-Golgi (Greenberg *et al.*, 1998). Another small hydrophobic protein, HTLV-1 p12ⁱ, which, like E5, binds to V-ATPase subunit c (Franchini *et al.*, 1993), causes the intracytoplasmic retention and degradation of MHC I (Johnson *et al.*, 2001). It thus appears that interference with either sector of the proton pump prevents MHC I localization to the cell surface, either in the exocytic or the endocytic pathway.

We observed that mutant forms of E5 defective for transformation are not capable of down-regulating either surface or total MHC I. Therefore, inhibition of MHC I in primary cells appears to be associated with full transformation, including malformation of Golgi structures. It is tempting to speculate whether the transport or the post-translational modification of cellular proteins involved in cell transformation are similarly affected. Cell surface receptors and adhesion molecules are post-translationally modified and transported to the plasma membrane through the GA (Bellis *et al.*, 1999; Rudd *et al.*, 1999); small GTPases of the ras family are either stably or transiently targeted to and post-translationally modified in the endomembranes (Apolloni *et al.*, 2000; Choy *et al.*, 1999; Erickson *et al.*, 1996; Noguchi *et al.*, 1998). Aberrant glycosylation of glycoproteins by Golgi enzymes often accompanies malignant transformation (Dennis *et al.*, 1999). Interference with any or all of the proper processing of these proteins could lead to cell transformation and explain the pleiotropic action of E5.

In conclusion, the down-regulation of cell surface MHC I by E5 may directly affect immune recognition and clearance of virally-infected cells. E5 protein is produced early in the infection process, in the virally-infected basal keratinocytes in the deep layers of the papilloma. Loss of MHC I expression in these cells would lead to a loss of immune recognition by class-I restricted effector cells, which could contribute to viral

persistence and the subsequent development of malignancy.

Materials and methods

Cell lines

Primary embryonic bovine cells (PalF) transformed by papillomavirus oncogenes, with or without E5 from BPV-1 or BPV-4 (1-E5 or 4-E5 respectively), or their mutant forms, have been described before (O'Brien *et al.*, 1999). In these cells, the combination of BPV-4 E7, E5 from either BPV-1 or BPV-4, and activated ras induces cell transformation but immortalization is conferred by HPV-16 E6 (Pennie *et al.*, 1993), as the BPV-4 genome does not encode an E6 protein (Jackson *et al.*, 1991). Briefly, normal parental cells are designated PalF or 'parental'; cells transformed by E6, E7 and activated ras are designated 'no E5' and referred to in the text as 'control', and cells transformed as above but with the addition of either E5 protein are designated 'E5'. NIH 3T3 mouse fibroblasts transformed by BPV-4 E5 (3T3 4-E5) have also been previously described (O'Brien and Campo, 1998). Control NIH3T3 cells containing the empty expression vector are designated '3T3'. All cell lines were grown in Dulbecco modified Eagles medium (DMEM) (Life Technologies, UK) containing 10% foetal calf serum (FCS) at 37°C in 5% CO₂.

Construction of recombinant amphotropic retroviruses

The E5 ORF of BPV-4 was amplified by PCR and cloned into the *Bam*HI–*Hind*III site of the retroviral vector pLZRSpBMNZ (Pear *et al.*, 1993). The BPV-4 E5 ORF has at its 5' end a 39 bp sequence encoding the HA epitope (O'Brien *et al.*, 1999). The construct was propagated in the Max Efficiency STBL2 bacterial strain (Life Technologies). Transformations were carried out according to the manufacturer's guidelines and the plasmid was prepared according to the Plasmid Maxi Kit (Qiagen) protocol.

The plasmid was transfected into the packaging cell line Phoenix A (Mann *et al.*, 1983) using the Dotap[®] Liposomal method (Boehringer Mannheim). Briefly 3–5 × 10⁶ cells were seeded into medium flasks in DMEM/10% FCS and incubated for 24 h at 37°C in 5% CO₂. The cells were then transfected with 10 µg of plasmid DNA in OPTIMEM reduced serum medium (Life Technologies) and incubated at 37°C for 12 h. After replacing the medium, the cells were incubated for a further 12 h followed by 48 h at 33°C. The supernatant containing the packaged retrovirus was collected and centrifuged to eliminate cell debris. The supernatant was either used directly to infect the target cells or aliquoted and snap frozen for later use.

Infection of PalF cells with recombinant retroviruses

PalF cells (2 × 10⁵) were seeded into a 25 cm² culture flask and incubated at 37°C. Before infection, the medium was removed and cells were overlaid with equal volumes of viral supernatant and 2 × Infection Mix (10 µg/ml polybrene in DMEM, 10% FCS), in a total volume of 1.5 ml. The flasks were centrifuged at 500 g for 45 min, then incubated at 33°C for 6 h, when fresh DMEM, 10% FCS, was added to a final volume of 5 ml. Forty-eight hours or 5 days post infection the cells were harvested for investigation of GA morphology or flow cytometry analysis.

Visualization of Golgi apparatus

The GA was visualized by immunofluorescent detection using monoclonal antibody (mAb) 4A3 raised against GM130, an integral GA protein (Barr *et al.*, 1998). Cells were grown until 80% confluence in single well chamber slides. After removal of medium, the cells were washed twice with PBS and fixed with fresh 3% PFA in PBS for 20 min at room temperature. After the PFA fixation, a second fixation was performed by dipping the chamber slides in –20°C methanol for 4 min. The cells were washed three times in PBS, then incubated with mAb 4A3 for 30 min at room temperature and washed as above. The cells were then incubated with AlexaFluor[™] 488 goat anti-mouse IgG(H+L) conjugate (Molecular Probes) (1:1500 dilution) for 30 min at room temperature. Following a final wash with PBS, the slides were mounted in PBS and analysed under a fluorescence microscope.

Detection of MHC class I and transferring receptor by FACS

Cells were grown in a 175 cm² flask to approximately 80% confluence. After removal of the medium, the cells were washed once with PBS, then detached from the flask with trypsin/EDTA and pelleted at 200 g at room temperature for 5 min. The cell pellet was resuspended in DMEM, 10% FCS, for 1 h at 37°C to allow surface antigens to be re-expressed. The cells were washed and re-suspended in PBS, 1% BSA (PBS-B) at 10⁷ cells/ml. For the detection of surface MHC I, 100 µl of cells were aliquoted and incubated with an equal volume of anti-bovine MHC class I monomorphic mAb (IL-A19; Bensaid *et al.*, 1989) for PalF cells, and anti-mouse H-2L^d mAb (CL9011-A, Cedarlane Laboratories) for NIH 3T3 cells, for 30 min at 4°C. For the detection of surface transferrin receptor, cells were incubated with mAb IL-A165 (Naessens and Davis, 1996). The cells were washed three times in PBS-B, then gently resuspended in 100 µl of PBS-B and incubated with an equal volume of a 1:200 dilution of anti-mouse IgG-FITC (Sigma) at 4°C for 30 min in the dark. The cells were washed and resuspended in 500 µl PBS-B and analysed by flow cytometry. If the flow cytometry analysis was not carried out immediately, the cells were resuspended in 500 µl of 3% PFA in PBS and kept at 4°C.

For the detection of intracellular MHC I, the cells were fixed in 3% PFA in PBS for 20 min at room temperature, washed in PBS-B and permeabilized with 0.5% saponin in PBS-B for 30 min at room temperature. Following a further wash in PBS-B, the permeabilized cells were stained with mAb IL-A19 as described above. Samples were examined in a Beckman Coulter EPICS Elite analyser equipped with an ion argon laser with 15 mV of excitation at 488 nm. The data were analysed using Expo 2 software.

Detection of MHC I by Western blots

Cells were removed from the flasks by trypsinization, washed with PBS, then lysed by sonication in lysis buffer (100 mM Tris HCl, pH 7.5, 2% SDS, 20% glycerol) and insoluble material was removed by centrifugation at 20000 g. Two, 5 and 10 µg of lysate were electrophoresed in 4–12% NuPAGE gels (Invitrogen), and proteins transferred to nitrocellulose membrane (Invitrogen) using a semidry blotting apparatus at 20 v/150 A for 1 h. The membranes were blocked in 5% milk/TBS/Tween 20 (0.05%) at room temperature for 1 h before incubation with mAb IL-A88 which recognizes MHC I heavy chain (Toye *et al.*, 1990) or mAb AB-1 (Calbiochem) specific for bovine actin. After repeated washing with TBS/Tween 20 (0.05%) the mem-

branes were incubated with anti-mouse Ig-HRP (Amersham Pharmacia Biotech) for mAb IL-A88, and anti-mouse IgM-HRP (Oncogene Calbiochem-Novabiochem International) for mAb AB-1, in 5% milk/TBS/Tween 20 (0.05%) for 1 h at room temperature. The membranes were washed three times with TBS/Tween 20 (0.05%) and bound antibody was detected by enhanced chemoluminescence staining (ECL) (Amersham Pharmacia Biotech).

Detection of MHC I heavy chain transcripts by RT-PCR

RNA was extracted from normal parental PalF cells and transformed cells expressing either 1-E5 or 4-E5 using the 'RNeasy[®] Mini kit' (Qiagen) according to the manufacturer's guidelines. Reverse transcription and amplification was carried out in one step using the 'Superscript One-Step RT-PCR' kit (Life Technologies). The MHC I-generic primers BoLaex2F (5'-GGC TCC CAC TC(G/C) (A/C)TG AGG TAT TTC) and BoLaex3R (5'-TCT CCA GGT ATC TGC GGA GCC) were designed to amplify exons 2 and 3 of all classical MHC I heavy chain sequences generating a product of 533 base pairs (bp) from RNA and a 733 bp product from DNA (Sawhney et al., 1995). As a positive control, bovine actin primers were used which generate a 273 bp fragment (Sibbet et al., 2000).

The RT-PCR reactions were carried out in 50 µl total volume, MHC I primers were used at a final concentration of 0.2 µM, bovine actin primers at 0.5 µM and RNA was adjusted to 1 µg. A semi-quantitative approach was used where reactions underwent the same RT-cycle (45°C for

30 min followed by inactivation of the reverse transcriptase at 94°C for 2 min), but the amplification cycles (94°C for 30 s, 55°C for 1 min, 72°C for 1 min) differed by multiples of 5, from 10 to 30 cycles. Fifteen µl of each reaction were run on a 2% agarose gel alongside a 100 bp DNA ladder (Life Technologies) and the amplicon bands were visualized by ethidium bromide staining.

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Appendix (b)

The bovine papillomavirus oncoprotein E5 retains MHC class I molecules in the Golgi apparatus and prevents their transport to the cell surface

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During papillomavirus infection, the E5 protein localizes in the cell Golgi apparatus and other endomembrane compartments. Cells transformed by E5 do not express major histocompatibility class I complex (MHC I) on the cell surface, while cells transformed by the other transforming proteins E6 and E7 do. In addition, the total amount of both MHC I protein and mRNA is reduced in E5-transformed cells. Here we show that expression of bovine papillomavirus E5 causes the retention of MHC I in the Golgi apparatus, thus preventing its transport to the cell surface. We ascribe this effect to a failure of acidification of the Golgi apparatus, as similar effects are observed in control cells treated with the ionophore monensin. Treatment of E5-transformed cells with either β - or γ -interferon increases the synthesis of MHC I, showing that inhibition of MHC I expression by E5 is not irreversible. However, even after interferon treatment, MHC I, although increased in quantity, is not transported to the cell surface. E5 therefore affects MHC I at several levels, but prevention of MHC I transport to the cell surface appears to be the dominant effect. Lack of surface MHC I would have profound consequences for presentation of viral peptides to the immune system.

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Keywords: papillomavirus; E5; MHC class I; immune evasion; Golgi apparatus

Introduction

Papillomaviruses (PV) are small DNA tumour viruses which infect mucosal and cutaneous epithelia where they induce benign hyperproliferative lesions. In most cases, papillomavirus infections are cleared after several months by a cell mediated immune response directed against viral antigens (Frazer and Tindle, 1996). Occasionally, however, the lesions do not regress; failure of virus clearance is attributed to a poor immunological response and persistent viral infection poses a greater risk of neoplastic progression.

Together with E6 and E7, E5 is a transforming protein of PV. While E6 and E7 are the main oncoproteins of mucosal human papillomavirus (HPVs) (Mantovani and Banks, 2001; Munger *et al.*, 2001), E5 is the major oncoprotein of bovine papillomaviruses (BPVs), particularly of BPV-1 (DiMaio and Mattoon, 2001). The E5 protein is a small hydrophobic peptide (from 83 amino acid residues in HPV-16 to 42 residues in BPV-4), and recent data suggests that it is a type II membrane protein with one α -helical trans-membrane span in BPV E5 and up to three trans-membrane spans in HPV E5 (Surti *et al.*, 1998). It is expressed during the early stages of infection in the deep layers of the infected epithelium and its expression is extinguished as the lesion progresses (Burnett *et al.*, 1992; Anderson *et al.*, 1997; Chang *et al.*, 2001).

In agreement with its hydrophobic nature, E5 is localized in the endomembrane compartments of the endoplasmic reticulum (ER) and Golgi apparatus (GA) of the host cell (Burkhardt *et al.*, 1989; Pennie *et al.*, 1993). The function of E5 *in vivo* is not known, but *in vitro* cell transformation is brought about by the activation of several kinases, from growth factor receptors to cyclins-cdks (Morgan and Campo, 2000). E5 interacts physically with the cellular protein 16k ductin/subunit c, a component of the gap junction and of the V0 sector of the vacuolar H⁺-ATPase (Goldstein *et al.*, 1991; Conrad *et al.*, 1993; Finbow *et al.*, 1995; Faccini *et al.*, 1996). This physical interaction has been deemed responsible for the down-regulation of gap junction communication (Oelze *et al.*, 1995; Faccini *et al.*, 1996; Ashrafi *et al.*, 2000) and for the lack of acidification of endosomes and GA (Straight *et al.*, 1995; Schapiro *et al.*, 2000). The inhibition of acidification of the endomembrane components has been attributed to a malfunction of the H⁺-ATPase (Briggs *et al.*, 2001), although lack of acidification of late endosomes or inhibition of the vacuolar proton pump are not always observed (Thomsen *et al.*, 2000; Ashby *et al.*, 2001).

We have recently shown that transformed cells expressing BPV-1 or BPV-4 E5 are highly vacuolated (Ashrafi *et al.*, 2000) and have an enlarged and often disrupted GA (Ashrafi *et al.*, 2002), and have attributed this morphology to the interaction of E5 and 16k subunit c. In these cells, E5 proteins, including E5 from HPV-16, cause down-regulation of the major

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histocompatibility class I (MHC I) complex (Ashrafi *et al.*, 2002; O'Brien and Campo, 2002), and this may contribute to PV escape from immunosurveillance and aid the establishment of viral infection.

Here we extend our observations on MHC I down-regulation in BPV E5-transformed cells and report that E5 causes the retention of MHC I in the GA and prevents its export to the plasma membrane. The lack of surface expression of MHC I is observed even when the levels of MHC I heavy chain are increased by interferon treatment of E5 cells. The interference of E5 with MHC I transport is likely due to the lack of acidification of the GA in E5 cells, as similar effects are observed in non-transformed and non-E5 transformed control cells treated with the ionophore monensin.

Results

MHC I is retained in the GA in E5-transformed cells

MHC I is consistently absent from the cell surface in cells transformed by BPV-1 E5 (1-E5) or BPV-4 E5 (4-E5), as assessed by FACS analysis (Figure 1a) (Ashrafi *et al.*, 2002). To ascertain the intracellular localization

of MHC I in E5 cells, primary bovine cells (PalF), PalF cells transformed by E6 and E7 (designated no-E5) and by the addition of E5 (designated E5 cells) were stained with either mAb IL-A19 or IL-A88 (Bensaid *et al.*, 1989; Toye *et al.*, 1990), which recognize β 2-microglobulin-associated MHC I heavy chain or free heavy chain, respectively. In PalF and no-E5 cells, MHC I was detected on the cell surface and in a Golgi-like structure (Figure 1b). In contrast in both 1-E5 and 4-E5 transformed cells, MHC I was detected only in abnormal structures (Figure 1b), reminiscent of the misshapen GA in these cells (Ashrafi *et al.*, 2002). Similar results were obtained with either antibody (data not shown).

E5 and MHC I heavy chain co-localize in the GA

To define the intracellular structures in which MHC I was detected, PalF, no-E5 and 4-E5 cells were transfected with a plasmid encoding a fusion of green fluorescent protein (GFP) and MHC I heavy chain, and stained with the Golgi marker BODIPY-TR-ceramide. In all cell lines, GFP-MHC I heavy chain localized in the GA, which had the normal 'ring-like'

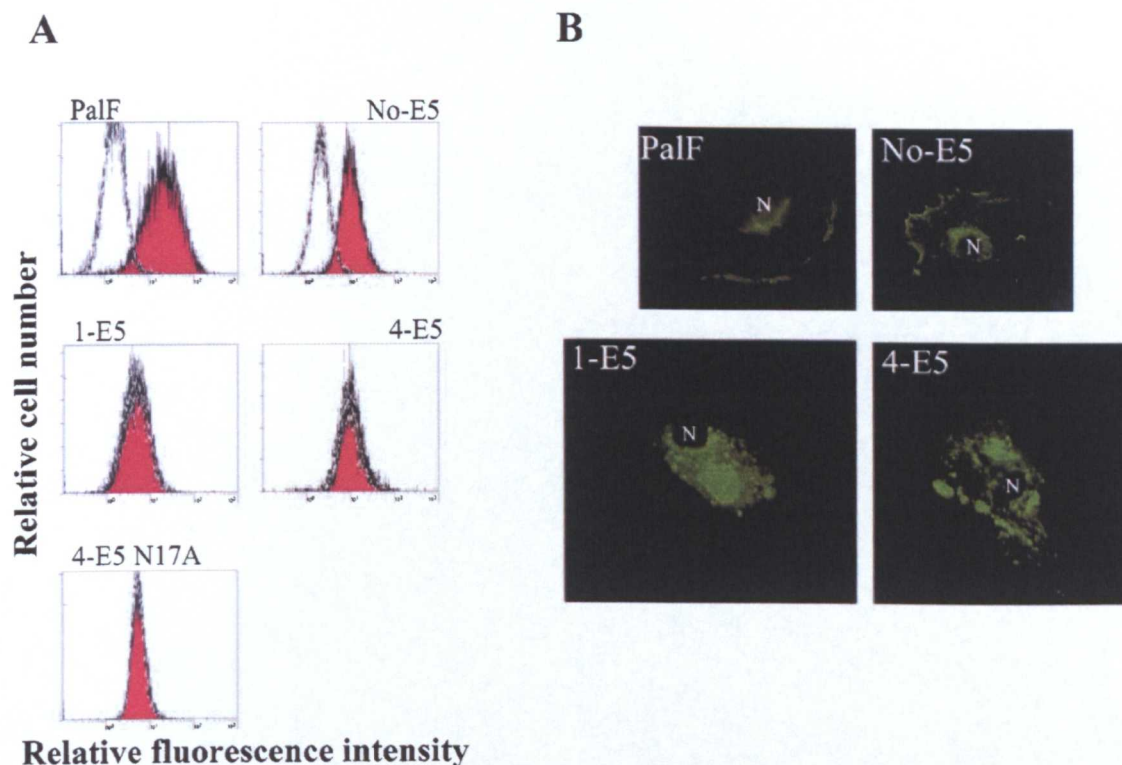


Figure 1 MHC class I is absent from the cell surface of E5-transformed cells. (a) FACS profiles of MHC I surface expression in parental (PalF) cells, transformed control cells (no-E5), and cells transformed by BPV-1 E5 (1-E5), BPV-4 E5 (4-E5) or the 4-E5 hypertransforming mutant N17A (Ashrafi *et al.*, 2000). Cells were stained with mAb IL-A19. The open histograms represent cells stained with FITC-conjugated secondary antibody only. Control cell lines express surface MHC I (solid histograms), whereas E5-transformed cells do not as indicated by the almost complete overlap of the two histograms. (b) Immunofluorescence detection of MHC I in PalF, no-E5, 1-E5 and 4-E5 cells. MHC I heavy chain was detected on the cell surface and in GA-like structures in parental and control cells, but only in GA-like structures in E5 cells. No fluorescence was detected in cells reacted only with the secondary antibody (data not shown). N = nucleus

appearance in PalF and no-E5 cells, whereas it was swollen and distorted in 4-E5 cells (Figure 2a).

To investigate whether E5 and MHC I co-localize, the cell lines were co-transfected with GFP-MHC I heavy chain and a plasmid encoding a fusion protein of the fluorescent protein DsRed and BPV-4 E5 (RFP-E5). Like wild type E5 (Pennie *et al.*, 1993), RFP-E5 localized in the GA (Figure 2b), whereas DsRed on its own was distributed uniformly in the nucleus and cytoplasm (data not shown). In all cell lines, GFP-MHC I heavy chain co-localized with RFP-E5 in the GA (Figure 2b).

Retention of MHC I in the GA by E5 is specific

It was important to demonstrate that retention of MHC I in the GA was specific, and not due, for instance, to the nature of the fusion proteins used in the study. Therefore, the cells were transfected with a plasmid expressing the papillomavirus E2 protein. As expected (Ham *et al.*, 1991), E2 localized in the nucleus in all cell lines, including 4-E5 transformed cells (Figure 3a), showing that E5 did not retain a predominantly nuclear protein in the endomembrane compartment. Moreover, co-expression of E2 and GFP-MHC I heavy chain, or E2 and RFP-E5 did not lead to co-localization: E2 was nuclear in all cases, and MHC I heavy chain and E5 were localized in the GA (Figure 3b).

Prevention of GA acidification by the ionophore monensin prevents transport of MHC I to the cell surface

The monovalent ionophore monensin is widely used to investigate the function of the GA and vesicular transport (see Barchet *et al.*, 2001; Halaban *et al.*, 2002; Schoonderwoert *et al.*, 2002). Monensin

disperses the proton gradient across the cell membranes and impedes the proper acidification of Golgi cisternae by displacing and inhibiting the H^+ -V-ATPase proton pump, leading to GA swelling and fragmentation and faulty protein transport from the medial to the *trans*-Golgi (Tartakoff, 1983; Boss *et al.*, 1984; Zhang *et al.*, 1996; Chikuma *et al.*, 2002). E5 complexes with 16k subunit c, a component of the V0 sector of the H^+ -V-ATPase (Goldstein *et al.*, 1991; Conrad *et al.*, 1993; Faccini *et al.*, 1996; Ashrafi *et al.*, 2000) and inhibits endomembrane acidification (Straight *et al.*, 1995; Schapiro *et al.*, 2000). In view of the similarity between the reported morphology of monensin-treated cells and E5 cells, and the functional similarity between monensin and E5, we treated PalF and no-E5 cells with monensin and analysed them for morphology, GA architecture and surface MHC I. Indeed, monensin-treated PalF cells or no-E5 cells showed extensive vacuolization and a grossly deformed GA (Figure 4a, shown only for PalF cells). These results indicate that the vacuolization and GA malformation in E5 cells are likely due to the impeded acidification of the GA membranes brought about by the viral protein.

Next we investigated whether treatment with monensin resulted in a down-regulation of surface MHC I in either PalF or no-E5 cells. Cells were treated with 25 μ M monensin for 30 min, 3 or 12 h, and analysed for surface or total MHC I by FACS. Monensin treatment caused a fourfold decrease in surface MHC I after 30 min in PalF cells and after 3 h in no-E5 cells (Figure 4b). The levels of surface MHC I returned to those seen in non-treated cells after 12 h in both cell lines (data not shown) in agreement with the reported ability of cells to recover from the effects of monensin treatment (Zhang *et al.*, 1993). We do not know why no-E5 cells down-regulated surface MHC I

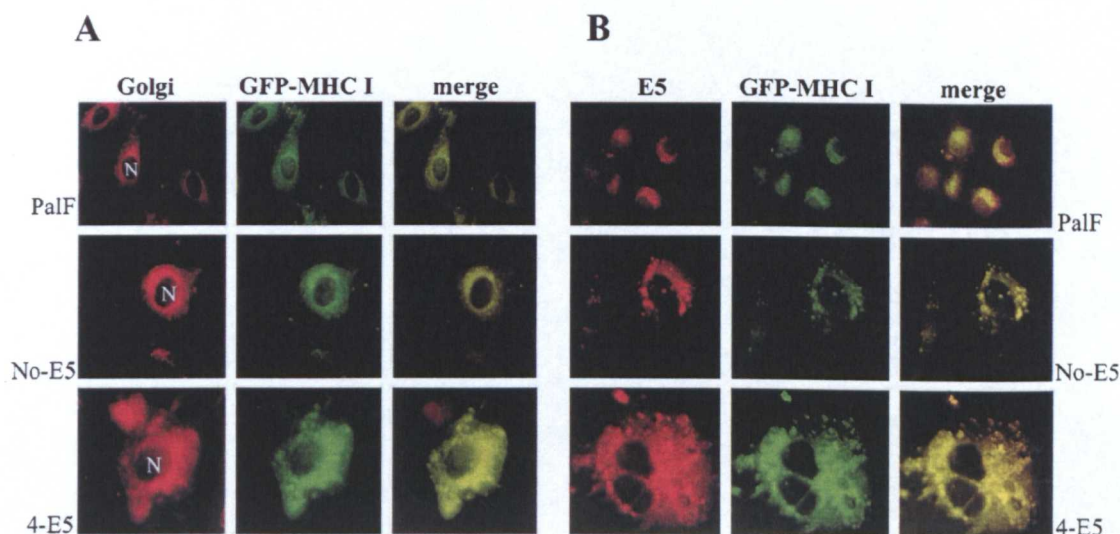


Figure 2 MHC I heavy chain and E5 co-localize in the GA. (a) Cells stained with BODIPY-TR-Ceramide (red) to detect the GA and transfected with GFP-MHC I heavy chain (green). GFP-MHC I heavy chain localizes in the GA in all cell lines. (b) Cells transfected with RFP-E5 (red) or GFP-MHC I heavy chain (green). MHC I heavy chain co-localizes with E5 in the GA in all cell lines

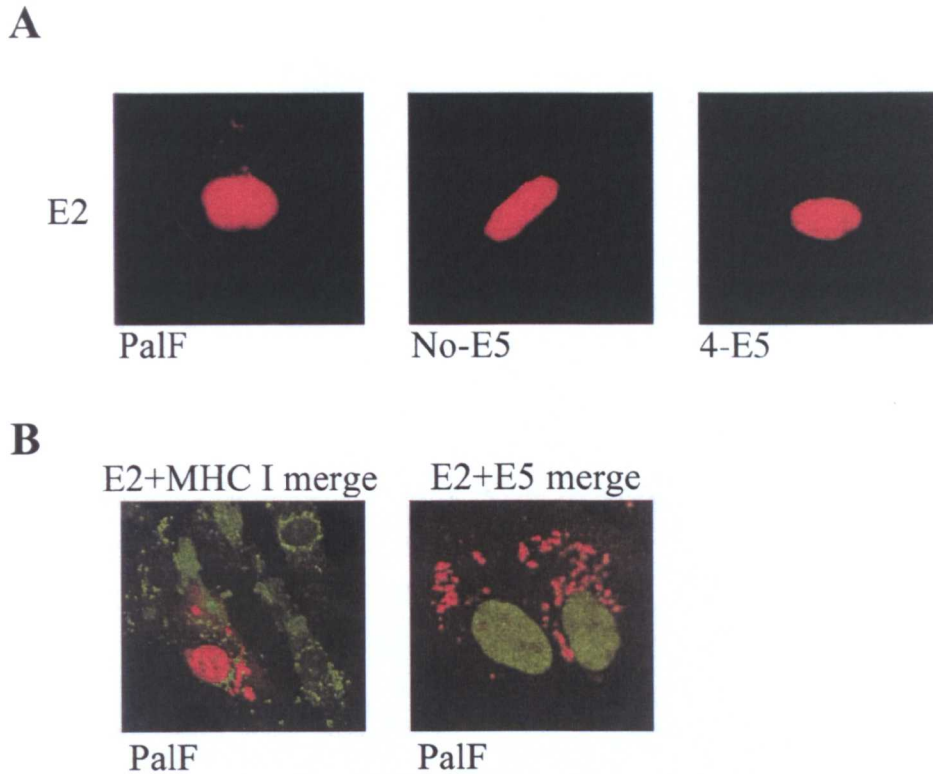


Figure 3 E2 localizes to the nucleus and does not co-localize with either MHC I heavy chain or E5. (a) Cells were transfected with E2 only, or (b) co-transfected with E2 and GFP-MHC I or E2 and RFP-E5. E2 was detected with mAb TVG216 and Texas Red-conjugated secondary antibody in (a) and in the left panel of (b), and with a FITC-conjugated secondary antibody in the right panel of (b). GFP-MHC I and RFP-E5 were visualized directly

in response to monensin less rapidly than PalF cells (3 h vs 30 min); the delay may be due to the expression of the other oncoproteins present in no-E5 cells. The experiment was repeated three times and the ratio between the mean fluorescence for total and surface (T/S) MHC I was calculated. The T/S ratio confirmed that monensin treatment reduces expression of surface MHC I: after 30 min of monensin treatment, the T/S ratio in PalF cells was increased approximately four-fold, and in no-E5 cells the highest T/S ratio was obtained after 3 h of treatment (Figure 4b). The transport to the cell surface of another membrane glycoprotein, the transferrin receptor, was not affected in E5 cells (Ashrafi *et al.*, 2002), and likewise there were no differences in the levels of surface transferrin receptor between cells treated and not treated with monensin (data not shown).

Monensin treatment did not significantly affect the levels of total MHC I in PalF cells and slightly decreased it in no-E5 cells, as judged by immunoblotting (Figure 4c), and therefore we conclude that monensin inhibited the transport of MHC I to the cell surface but not its expression. These results indicate that, following inhibition of GA acidification by monensin, transport of MHC I to the cell surface is impeded, and support the notion that E5 prevents MHC I transport through inhibition of GA acidification.

Treatment of E5 cells with interferon increases total but not surface MHC I

Both β -interferon and γ -interferon (β -IFN or γ -IFN) increase the transcription activity of the MHC I heavy chain gene promoter (Agrawal and Kishore, 2000) leading to higher expression levels of heavy chain. We have previously shown that in E5 cells there is less MHC I heavy chain mRNA and protein (Ashrafi *et al.*, 2002). To see if the biosynthetic pathway of heavy chain is permanently inhibited by E5, we treated PalF, no-E5 and E5 cells with either β - or γ -IFN or the control supernatant 2DG. Treatment with either IFN, but not with 2DG, noticeably increased production of the MHC I heavy chain to approximately the same extent in all cell lines as assessed by immunoblotting (Figure 5a). This shows that E5 cells are responsive to IFN and the biosynthetic pathway of MHC I heavy chain is not irreversibly inhibited by E5. The IFN-treated cells were also analysed by FACS to ascertain whether the increased production of heavy chain led to a corresponding increase in surface MHC I. MHC I surface expression increased only in PalF and no-E5 cells but not in E5 cells, or in cells expressing the hypertransforming 4-E5 N17A mutant (Figure 5b), despite the increase in total MHC I. These results unequivocally show that E5 inhibits the transport of

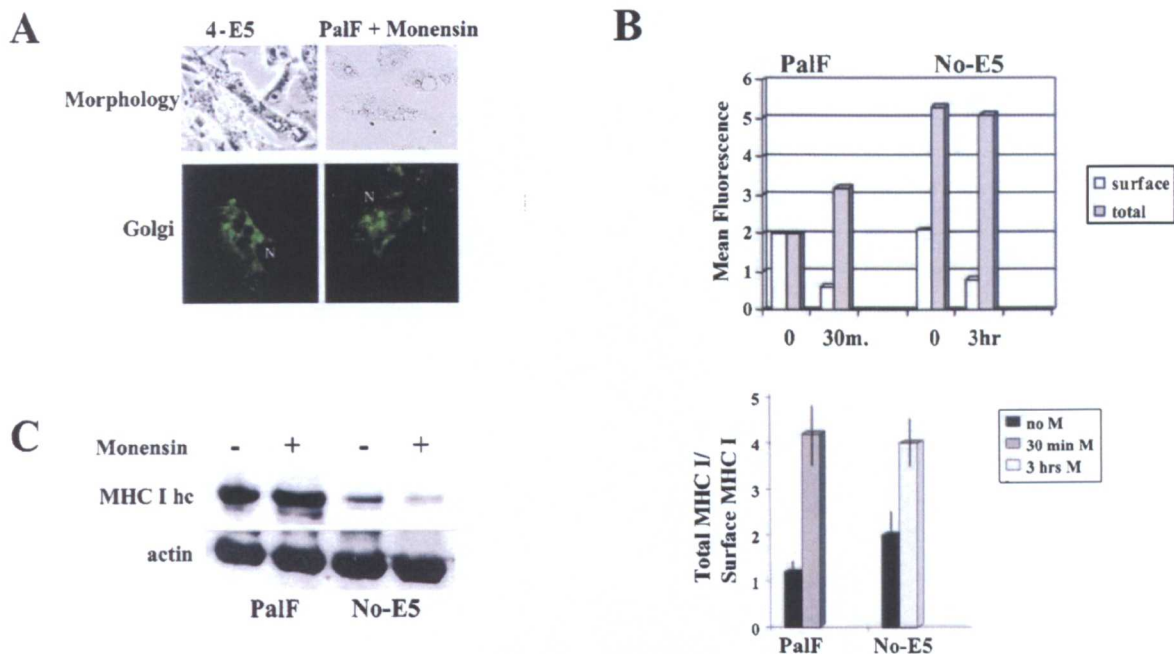


Figure 4 Monensin causes GA swelling and MHC I intracellular retention. (a) Morphology of 4-E5 cells and PalF cells treated with 25 μ M monensin for 3 h using phase contrast microscopy (top panels), and immunofluorescence with mAb 4A3 to visualize the GA (bottom panels). Monensin-treated cells are highly vacuolated and present a swollen GA, similar to E5 cells, N=nucleus. (b) PalF and no-E5 cells were treated with 25 μ M monensin for 30 min or 3 h. Surface and total MHC I expression was examined by FACS analysis using mAb IL-A19. The mean fluorescence from a representative experiment is shown in the upper panel. Surface mean fluorescence decreases after 30 min treatment with monensin in PalF cells and after 3 h treatment in no-E5 cells. The ratio between total mean fluorescence and surface mean fluorescence (T/S) of PalF and no-E5 cells is shown in the bottom panel. The bars represent the average ratio from three experiments (\pm standard deviation). The T/S ratio increases approximately fourfold in PalF cells after 30 min of monensin treatment, and approximately twofold in no-E5 cells after 3 h of treatment, indicating a decrease in surface MHC I. (c) PalF and no-E5 cells were treated with 25 μ M monensin for 3 h and 10 μ g of protein lysate were analysed by immunoblotting for MHC I expression. MHC I heavy chain (MHC I hc) was detected with mAb IL-A88 (top panel) and actin, as a loading control, with mAb AB-1 (bottom panel). Monensin does not affect the expression of MHC I heavy chain

MHC I from the endomembrane compartment to the cell surface.

Discussion

MHC class I molecules play a crucial role in immune recognition and clearance of virus infected cells. The proteolytic degradation of viral proteins generates a spectrum of peptides that are transported into the ER and loaded onto the MHC class I complex, which consists of the MHC I heavy chain and β 2-microglobulin. Following transport from the ER through the GA to the cell surface, the MHC class I/peptide complexes are presented to virus-specific cytotoxic T lymphocytes (CTL), signalling virus infection and enabling killing of the infected cell. The importance of MHC class I molecules in virus clearance has been demonstrated by the identification of many mechanisms of interference with the MHC class I pathway evolved by viruses, from prevention of peptide processing to formation of non-functional MHC I complexes (Yewdell and Bennink, 1999). In each of these strategies, the outcome is the failure by the infected cell to present viral peptide antigens to effector

T cells and hence avoidance of detection and destruction by the host immune system.

PV are poorly immunogenic. Even in immunocompetent hosts, whether animals or humans, PV persist for a significant period of time, usually spanning several months, before activation of the host immune system. This lack of recognition, in spite of the expression of abundant viral protein, suggests the host immune system is unaware of, or disabled by, PV infection. The ability of the virus to persist has been attributed to the nature of the virus life cycle, which may prevent the immune cells having access to the viral proteins (Tindle, 2002). Although undoubtedly a factor in immune evasion, there is now increasing evidence that PV encode proteins that can directly subvert the host immune response, resulting in a delay in, or prevention of, resolution of infection (O'Brien and Campo, 2002). We have recently shown that the PV oncoprotein E5 can down-regulate the surface expression of MHC I, which may affect the recognition and killing of PV-infected cells by CTL (Ashrafi *et al.*, 2002; O'Brien and Campo, 2002). E5 appears to interfere with several steps of the MHC I pathway, including transcription of MHC I heavy chain mRNA, expression of MHC I heavy chain protein, and

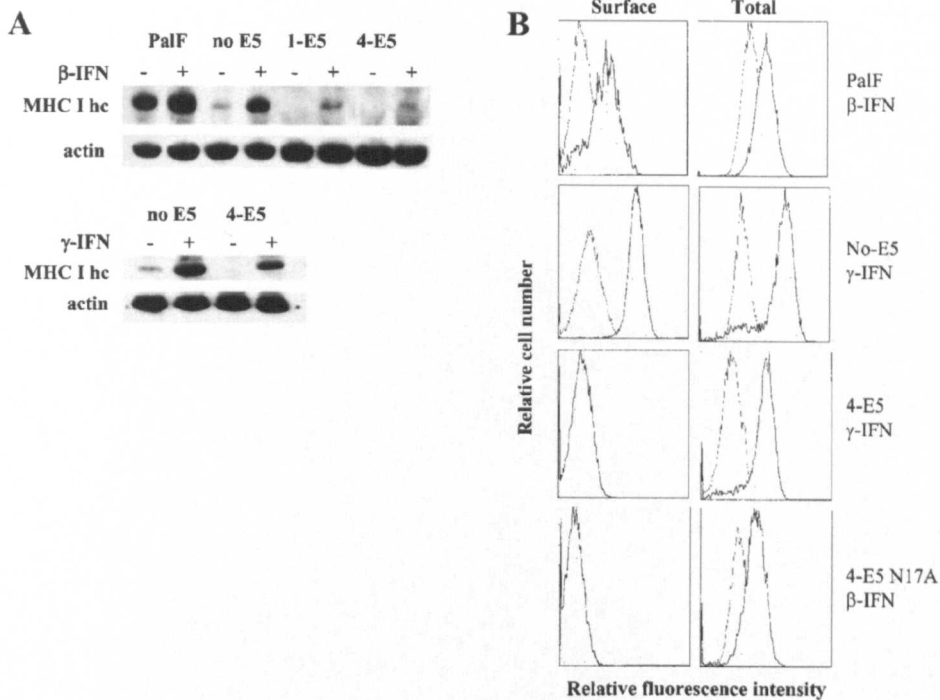


Figure 5 (a) β -IFN and γ -IFN increase MHC I heavy chain expression in all cells (a). Cells were treated for 48 h with 500 U/ml of either β -IFN or γ -IFN (lanes marked +), or with 500 U/ml of the control supernatant 2D6 (lanes marked -), and MHC I was analysed by immunoblotting as described in Figure 4. (b) MHC I is not transported to the cell surface in IFN-treated E5 cells. Cells treated with β -IFN (PalF, 4-E5 N17A) or γ -IFN (no-E5, 4-E5) (solid histograms), or the control supernatant 2D6 (open histograms) were analysed for expression of total and surface MHC I by FACS analysis with mAb IL-A19. IFN treatment increases both total and surface MHC I in PalF and no-E5 cells, but only total MHC I in E5 cells or cells expressing the hypertransforming mutant N17A

transport of MHC I complex to the plasma membrane (Ashrafi *et al.*, 2002). Here we have focused our attention on the faulty transport of MHC I to the cell surface.

In order to determine if the MHC I biosynthetic pathway was rendered irretrievably malfunctioned by E5, we treated the cells with type I and II IFNs which are potent inducers of MHC I expression (Agrawal and Kishore, 2000). All of the cell lines tested, including the E5 cells, responded to IFN treatment by increasing the level of MHC I heavy chain to approximately the same extent, although the effect was more marked with γ -IFN than with β -IFN. Thus, although E5 reduces the levels of both mRNA and protein, transcription and translation of the MHC I heavy chain can be recovered showing that the effects of E5 on MHC I expression are not irreversible. What is clearly irreversibly impaired in E5 cells is the transport of MHC I to the cell periphery, as despite the marked increase in MHC I heavy chain, no MHC I is found on the surface of the IFN-treated E5 cells. E5 therefore retains MHC I intracellularly.

Both endogenous MHC I or exogenous (transfected) GFP-MHC I heavy chain fusion protein are retained in the swollen and misshapen GA characteristic of E5-expressing cells and indeed co-localized with the E5 protein in the GA. The detection of endogenous MHC

I in the GA with mAb IL-A19, which does not recognize free MHC I heavy chain, indicates that the heavy chain is complexed with β 2-microglobulin. This suggests that the arrest of MHC I transport occurs following MHC class I complex formation in the ER and is instead a fault in cellular transport across the Golgi compartments. Indeed, treatment of control cells with the ionophore monensin resulted in the acquisition of a phenotype remarkably similar to that of E5 cells, with many vacuoles and a distorted GA, with a concordant rapid and marked down-regulation of surface MHC I. Monensin is a well-characterized inhibitor of GA acidification, which hinders cellular transport of newly synthesized proteins, particularly interfering with transfer across Golgi compartments and compromising secretion from the medial to the *trans*-Golgi (Tartakoff, 1983). Taking into account the similar functional effect of monensin and E5, we suggest that E5 causes GA malformation and retention of the MHC I complex in the GA by increasing GA pH.

However, the impeded transport of the MHC I complex in E5 cells is not part of a generalized disturbance of intracellular traffic as we have shown that the transport of the transferrin receptor is not affected by E5 expression (Ashrafi *et al.*, 2002) nor by treatment with monensin. How then does E5 prevent

MHC I from exiting the GA and reaching the plasma membrane?

Like E5, the HTLV-1 p12¹, another small hydrophobic viral protein, binds to the 16k subunit c component of the H⁺-V-ATPase (Franchini *et al.*, 1993). p12¹ can also bind MHC I causing its intracytoplasmic retention and degradation (Johnson *et al.*, 2001). However, we feel that a physical interaction between E5 and MHC I is an unlikely cause of MHC I retention in the GA. E5 is expressed at a very low level in transformed cells (O'Brien *et al.*, 1999), and although IFN treatment markedly increases the expression of MHC I heavy chain, it has no effect on E5 expression (R Ullah *et al.*, unpublished observations). Hence a one-to-one interaction between E5 and MHC I heavy chain is stoichiometrically incompatible.

A more likely explanation may be that E5-induced acidification of the GA causes the incorrect glycosylation of the MHC I heavy chain. This hypothesis is supported by the observation that monensin disrupts the processing of N- and O-linked carbohydrates on glycoproteins and this disruption inhibits the intracellular transport of a variety of proteins (Anderson and Pathak, 1985). Moreover, it has recently been reported that 16k subunit c suppresses the tumour-related glycosylation of cell surface receptors, and that this effect is independent of V-ATPase function (Skinner and Wildeman, 2001). Therefore, it is possible that the interaction of E5 with 16k subunit c may lead to improper glycosylation and processing of MHC I. Improperly processed MHC I molecules accumulate and are degraded in an expanded Golgi intermediate compartment (Raposo *et al.*, 1995), which is consistent with the cellular localization of the MHC class I complex in the E5 cells. In addition, degradation would account for the reduced amounts of MHC I heavy chain observed in E5 cells. It remains to be seen if inhibition of the E5-16k subunit c interaction can revert, at least partially, the transformed phenotype of E5 cells and restore surface MHC I expression.

Materials and methods

Cell lines

Primary embryonic bovine cells (PalF) transformed by PV E6 and E7 oncogenes, with or without BPV-1 or BPV-4 E5 (1-E5 or 4-E5 respectively), have been described previously (Ashrafi, 1998; O'Brien *et al.*, 1999). In these cells, the combination of BPV-4 E7, E5 from either BPV-1 or BPV-4, and activated ras induces cell transformation but immortalization is conferred by HPV-16 E6 (Pennie *et al.*, 1993), as the BPV-4 genome does not encode an E6 protein (Jackson *et al.*, 1991). Briefly, normal parental cells are designated PalF; cells transformed by E6, E7 and activated ras are designated 'no-E5' and cells transformed as above but with the addition of either E5 protein are designated 'E5'. The cell lines were grown in Dulbecco's modified Eagles medium (DMEM) (Invitrogen/Life Technologies, UK) containing 10% foetal calf serum (FCS) at 37°C in 5% CO₂, unless specified to the contrary.

Treatment of cells with monensin

PalF and no-E5 cells were treated with 25 µM monensin (Sigma, UK) for 30 min, 3 or 12 h. The morphology of the GA and surface MHC I were determined as described below.

Treatment of cells with interferon

One million PalF, no-E5 and E5-transformed cells were seeded in 100 mm diameter tissue culture dishes. The following day, the medium was replaced with fresh medium with or without 500 U/ml β-IFN (Sigma) or recombinant ovine γ-IFN (Graham *et al.*, 1995). 2D6, a supernatant from cells not expressing IFN, was used as control. After 48 h, the cells were harvested for detection of MHC I by immunoblotting or FACS analysis (see below). Ovine recombinant γ-IFN and 2D6 were a kind gift of Dr G Entrican (Moredun Research Institute, Penicuik, UK).

Plasmids

pGFP-MHC I expresses a fusion protein made up of the green fluorescent protein (EGFP) and the heavy chain of human MHC I B2705 and was a kind gift from Dr Simon Powis (University of Dundee, UK). pRFP-E5 expresses a fusion protein of the fluorescent protein DsRed and BPV-4 E5. It was constructed by inserting the BPV-4 E5 open reading frame tagged with the sequence for the HA epitope (O'Brien *et al.*, 1999) into the *Bgl*II and *Hind*III sites of pDsRed-C1 (Clontech, UK). pCMVE2 expresses the E2 protein of HPV-16 and was a kind gift from Dr Lawrence Bank (ICGEB, Trieste, Italy).

Antibodies

Monoclonal antibody (mAb) 4A3 is raised against GM130, an integral GA protein (Barr *et al.*, 1998) and was a kind gift of Dr Martin Lowe (University of Manchester, UK). MAbs IL-A19 and IL-A88 are raised against monomorphic determinant of bovine MHC I (Bensaid *et al.*, 1989; Toyé *et al.*, 1990), which recognize β2-microglobulin-associated MHC I heavy chain or free heavy chain, respectively. They are kind gifts of Dr Liz Glass (IAH, Roslin, UK) and Dr Shirley Ellis (IAH, Compton, UK), respectively. TVG261 recognizes the E2 protein of HPV-16 and was a kind gift of Dr Marilyn Hibma (University of Otago, New Zealand).

DNA transfection

Cells were aliquoted into 24-well plates containing coverslips at 10⁴ cells/well and grown overnight to approximately 80% confluence. Plasmids (0.6 µg) were transfected into cells using LipofectAMINE PLUSTM Reagent (Invitrogen/Life Technologies, UK) according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were washed twice with PBS and then fixed in 1.85% formaldehyde (Sigma) diluted in PBS containing 2% sucrose (Schwarz/Mann, USA) for 10 min at room temperature. Following a further three washes with PBS, the coverslips were removed from the well and mounted in AF1 (Citifluor, UK).

Visualization of GFP and RFP plasmids

Cells expressing fluorescent GFP-MHC I and RFP-E5 were visualized either using a Leica DMLB microscope or a Zeiss LSM 510 microscope.

Detection of E2

Cells were transfected with pCMVE2 and fixed 24 h after transfection as described above. Following washes in PBS, the cells were permeabilized in 0.5% NP40/PBS/10% sucrose for 10 min at room temperature and then washed three times with 1% FCS/PBS. The coverslips were incubated in mAb TVG261 for 1 h at room temperature, washed in 1% FCS/PBS and then incubated with an anti-mouse antibody conjugated either with Texas Red or with FITC (Sigma). Following further washes in 1% FCS/PBS, coverslips were mounted in AF1 and analysed as above.

Visualization of Golgi apparatus

The GA was visualized by staining with BODIPY-TR-ceramide. Cells were grown until 80% confluent in single well chamber slides. After removal of medium cells were washed twice with serum free DMEM, 25 mM HEPES (DMEM-H) and incubated in 200 μ l of 5 μ M BODIPY-TR-ceramide, which localizes to the GA, in DMEM-H for 30 min at 4°C. Cells were then washed with DMEM-H for 30 min at 37°C. After removal of the medium and washing with PBS, cells were fixed with fresh 3% paraformaldehyde (PFA) for 20 min at room temperature and mounted with Vectashield (Vector Laboratories Inc, UK).

Cytoimmunofluorescent detection of Golgi apparatus and MHC I

The cells were washed twice with PBS and fixed with fresh 3% PFA in PBS for 20 min at room temperature. After the PFA fixation, a second fixation was performed by dipping the chamber slides in -20°C methanol for 4 min; then the cells were washed three times in PBS. For GA detection, the cells were incubated with mAb 4A3 for 30 min at room temperature, washed as above and incubated with Alexa-FluorTM 488 goat anti-mouse IgG(H+L) conjugate (Molecular Probes, Europe BV) for 30 min at room temperature. For the detection of endogenous surface MHC I, the cells were incubated with mAb IL-A19 or mAb IL-A88 for 1 h at room temperature and washed three times as above. The cells were then incubated with anti-mouse IgG-FITC (Sigma) at 4°C for 1 h in the dark. Following three final washes with PBS, the slides were mounted in PBS and analysed under the fluorescence microscope.

Detection of MHC class I by FACS

Cells were grown in a 175 cm² flask to approximately 80% confluence. After removal of the medium, the cells were washed once with PBS, then detached from the flask with trypsin/EDTA and pelleted at 200 g for 5 min at room temperature. The cell pellet was resuspended in DMEM, 10% FCS, for 1 h at 37°C to allow surface antigens to be re-expressed. The cells were washed and re-suspended in PBS-

1% BSA (PBS-B) at 10⁷ cells/ml. For the detection of surface MHC I, 100 μ l of cells were aliquoted and incubated with an equal volume of mAb IL-A19 for 30 min at 4°C. The cells were washed three times in PBS-B and incubated with anti-mouse IgG-FITC (Sigma) at 4°C for 30 min in the dark. The cells were washed and resuspended in 500 μ l PBS-B and analysed by flow cytometry. If the flow cytometry analysis was not carried out immediately, the cells were resuspended in 500 μ l of 3% PFA in PBS and kept at 4°C.

For the detection of intracellular MHC I, the cells were fixed in 3% PFA in PBS for 20 min at room temperature, washed in PBS-B and permeabilized with 0.5% saponin in PBS-B for 30 min at room temperature. Following a further wash in PBS-B, the permeabilized cells were stained with mAb IL-A19 as described above. All samples were examined in a Beckman Coulter EPICS Elite analyser equipped with an ion argon laser with 15 mV of excitation at 488 nm. The data were analysed using Expo 2 software.

Detection of MHC I by immunoblotting

Cells were removed from the flasks by trypsinization, washed with PBS, then lysed by sonication in lysis buffer (100 mM Tris HCl, pH 7.5, 2% SDS, 20% glycerol) and insoluble material was removed by centrifugation at 20000 g. Ten μ g of lysate were electrophoresed in 4–12% NuPAGE gels (Invitrogen), and proteins transferred to nitrocellulose membrane (Invitrogen) using a semidry blotting apparatus at 20 V/150 A for 1 h. The membranes were blocked in 5% milk/TBS/Tween 20 (0.05%) at room temperature for 1 h before incubation with mAb IL-A88, or mAb AB-1 (Calbiochem) specific for actin. After repeated washing with TBS/Tween 20 (0.05%) the membranes were incubated with anti-mouse Ig-HRP (Amersham Pharmacia Biotech, UK) for mAb IL-A88, and anti-mouse IgM-HRP (Oncogene Calbiochem-Novabiochem International) for mAb AB-1, in 5% milk/TBS/Tween 20 (0.05%) for 1 h at room temperature. The membranes were washed three times with TBS/Tween 20 (0.05%) and bound antibody was detected by enhanced chemoluminescence staining (ECL) (Amersham Pharmacia Biotech).

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Appendix (c)

Short Communication

Downregulation of major histocompatibility complex class I in bovine papillomas

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Bovine papillomavirus (BPV) induces papillomas in cattle; in the great majority of cases, these regress due to the host immune response, but they can persist and progress to malignancy. Even in the absence of malignant transformation, BPV infection persists for a significant period of time before activation of the host immune system, suggesting that the host immune system is unaware of, or disabled by, BPV. E5 is the major oncoprotein of BPV, which, in addition to its transforming properties, downregulates the expression and transport to the cell surface of major histocompatibility complex class I (MHC I). Here, it is shown that co-expression of MHC I and E5 in papillomas caused by BPV-4 infection is mutually exclusive, in agreement with the inhibition of surface MHC I expression by E5 that is observed *in vitro*. The inhibition of MHC expression in E5-expressing papilloma cells could explain the long period that is required for activation of the immune response and has implications for the progression of papillomas to the malignant stage; absence of peptide presentation by MHC I to cytotoxic T lymphocytes would allow the infected cells to evade the host cellular immune response and allow the lesions to persist.

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Papillomaviruses are oncogenic viruses that induce benign proliferative lesions of epithelia, called papillomas or warts. Papillomavirus infections are usually eliminated by a cell-mediated immune response, which is directed against viral antigens (O'Brien & Campo, 2002). However, in a minority of cases, mucosal lesions do not regress and can progress to cancer. High-risk human papillomavirus types 16 and 18 (HPV-16 and -18) are the main causative factors in the development of cancer of the cervix uteri (zur Hausen, 2002). Bovine papillomavirus type 1 and 2 (BPV-1 and -2) and BPV-4 are respectively involved in carcinogenesis of the urinary bladder and the alimentary tract in cattle; BPV-1 is also involved in cancer of the penis (Campo, 1997, 2002). In all cases, cancer is preceded by, and derives from, pre-existing papillomas.

Papillomas that are induced by BPV develop through four well-defined stages, from stage 1 or plaque, the first clinical manifestation of infection, to stage 4, when the papilloma starts to regress (Jarrett, 1985). From infection to regression, papilloma development often takes longer than 12 months. In the case of BPV-4-induced papillomas, regression is accompanied by infiltration of immune cells, primarily CD4⁺ T lymphocytes in the adjacent dermis, as well as CD8⁺ T lymphocytes infiltrating the keratinocytes (Knowles *et al.*, 1996). Persistence, spread and progression of papillomas to cancer occur mainly in animals that graze on bracken fern (*Pteridium* spp.) (Campo *et al.*, 1994). These animals are immunocompromised by immunosuppressants, such as sesquiterpenes, that are present in the plant.

However, papillomas can also spread and persist in cattle that are not exposed to bracken fern (Tsirimonaki *et al.*, 2003). Even in the absence of malignant transformation, BPV infection can persist for a significant period of time before activation of the host immune system. Lymphocytes from infected animals do not recognize early or late viral antigens until late in infection, despite the presence of numerous papillomas actively producing virus (Chandrachud *et al.*, 1994, 1995; McGarvie *et al.*, 1995; Kirnbauer *et al.*, 1996). This lack of recognition suggests that the host immune system is unaware of, or disabled by, BPV infection. It is now known that papillomaviruses can subvert the immune response indirectly, as virus replication is confined to the epithelial cells above the basal membrane and therefore occurs in a site that is recognized poorly by immune cells (Frazer *et al.*, 1999). In addition, papillomaviruses appear to interfere directly with host antiviral immune mechanisms, including the interferon response and major histocompatibility complex class I (MHC I) antigen presentation to cytotoxic T lymphocytes (O'Brien & Campo, 2002; Tindle, 2002).

We have shown recently that expression of the E5 oncoprotein of BPV-1, BPV-4 and HPV-16 has a profound effect on the synthesis and transport of MHC I in cultured cells and, therefore, can potentially contribute to the ability of the virus to evade immune recognition (Ashrafi *et al.*, 2002, 2004; Marchetti *et al.*, 2002; O'Brien & Campo, 2002). To ascertain whether the downregulation of MHC I that is observed in cultured cells takes place in tumours, we

investigated the expression of BPV-4 E5 and MHC I in clinical samples of BPV-4 papillomas.

Papillomas from the palate, rumen and oesophagus, as well as samples of normal palate, tongue and buccal mucosa, were collected post-mortem from animals that were referred to the University of Glasgow Veterinary School. Tissue samples were fixed and stored in 10% formaldehyde in PBS at pH 7.5 and embedded in paraffin wax for histological processing. Serial sections (1.5 µm) were cut and placed on microscopic slides that had been treated with VECTABOND (Vector). After deparaffinization in Histo-clear (National Diagnostics), sections were rehydrated in graded ethanol and incubated in 0.5% H₂O₂/methanol for 20 min to quench endogenous peroxidase. Sections were subjected to antigen-retrieval treatment with 0.01 M sodium citrate buffer (pH 6) in a pressure cooker for 75 s at 103.4 kPa, blocked with 1% normal unlabelled swine serum (Scottish Antibody Production Unit) in TBS containing 0.1% Tween 20 for 30 min at room temperature and then incubated for 1.5 h at room temperature with primary antibodies for detection of E5, E7, MHC I heavy chain and the proliferation marker Ki67, as detailed below. The sections were then incubated with biotin-labelled secondary antibody (Dako)

and streptavidin-biotin complex (Dako) for 45 min, following the manufacturer's instructions. Immunoreactivity was visualized with diaminobenzene (Sigma). Sections were counterstained with Gill's haematoxylin, dehydrated, cleared in Histo-clear and mounted permanently with DPX mountant under a coverslip prior to microscopic examination. A total of seven papillomas was analysed, with at least three sections and three different section fields examined per papilloma; all papillomas were classified as stage 2/3, i.e. mature papillomas producing virus, and presented the typical features of BPV-4 infection (Jarrett, 1985), including an irregular basal layer (Fig. 1a and b), fronds of transformed cells terminating in keratinized tips (Fig. 2d) and koilocytes, cells with highly enlarged cytoplasm that are typical of papillomavirus infection (Fig. 2d, black arrow). In contrast, the normal bovine alimentary mucosal epithelium had a typical architecture, composed of a regular basal cell layer and supra-basal, spinous and squamous layers (Fig. 1d).

BPV-4 E5 was detected with each of two rabbit antisera, 274 and 275, that were raised against a synthetic peptide representing the 12 C-terminal amino acids of the protein, conjugated to keyhole limpet haemocyanin (Anderson *et al.*, 1997). Similar results were obtained with both anti-E5

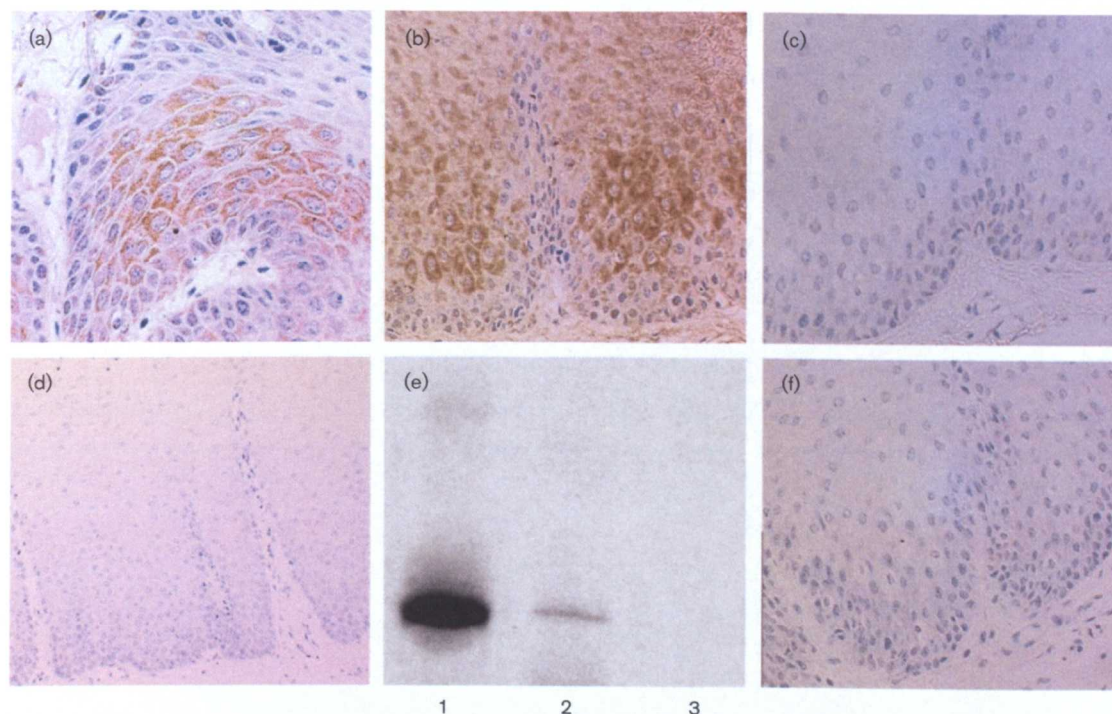


Fig. 1. Expression of E5 in BPV-4-induced papillomas. Representative papilloma sections stained with anti-E5 antiserum 274 (1:2000). (a) Papilloma 1803, showing E5 expression in basal and suprabasal layers. (b) Papilloma 386, showing E5 expression in suprabasal layers. (c) Serial section of papilloma 386 incubated with secondary antibody only. (d) Normal buccal mucosa, showing no expression of E5. (e) Autoradiograph of *in vitro*-labelled [³⁵S]E5. Lane 1, input E5 protein; lane 2, E5 immunoprecipitated with antiserum 274; lane 3, E5 immunoprecipitated with antiserum 274 pre-absorbed with an E5 C-terminal peptide. (f) Serial section of papilloma 386 incubated with pre-absorbed antiserum, showing loss of reactivity. Magnification, ×40 (a); ×20 (b, c and f); ×10 (d).

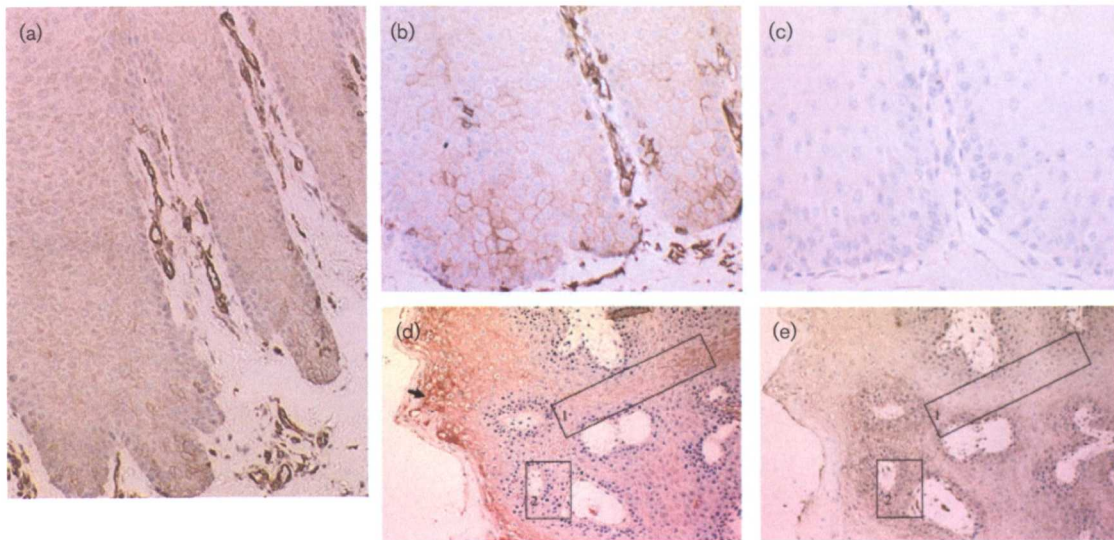


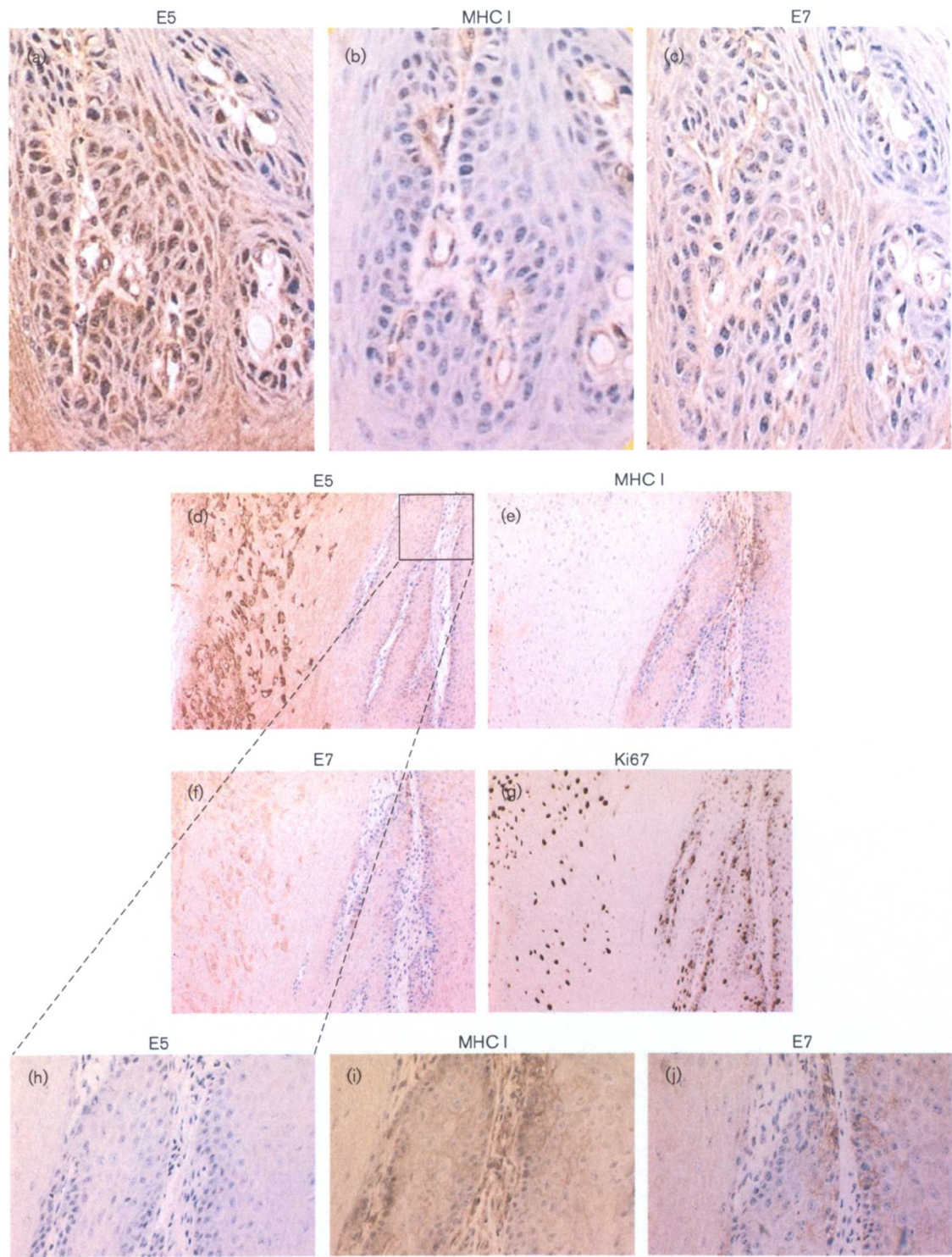
Fig. 2. Expression of MHC I in BPV-4-induced papillomas. Representative papilloma sections stained with anti-MHC I heavy chain mAb IL-A88 (1 : 200). (a) Section of normal buccal mucosa, showing MHC I expression throughout most of the thickness of the epithelium. (b) As (a) but at higher magnification, showing staining of MHC I on the cell surface. (c) Serial section of papilloma 386 stained with mAb IL-A88, showing lack of reactivity. (d) Section of papilloma 1804 stained with antiserum 274, showing E5 expression in the suprabasal, transit and lower spinous layers (boxed area 1) and in differentiated keratinocytes (black arrow). Boxed area 2 shows transit and lower spinous layers without E5 expression. (e) Serial section of papilloma 1804 stained with mAb IL-A88, showing MHC I expression in cells lacking E5 (boxed area 2) and lack of MHC I expression in cells expressing E5 (boxed area 1). Magnification, $\times 10$ (a, d and e) and $\times 20$ (b and c).

antisera; only results that were obtained with antiserum 274 are shown. In agreement with previous results by us and others (Burnett *et al.*, 1992; Anderson *et al.*, 1997), E5 was detected exclusively in the cytoplasm of epidermal cells, from the basal and parabasal layers to the spinous and squamous layers (Figs 1a and b, and 2d). Expression was, however, discontinuous, as reported previously (Anderson *et al.*, 1997); no papilloma was stained in all cell layers. To verify the specificity of the immunostaining of E5, several controls were carried out. There was no staining with pre-immune serum (data not shown) or when only secondary antibody was used (Fig. 1c), and the E5 antiserum did not react with normal mucosa (Fig. 1d). Furthermore, when the E5 antiserum was pre-absorbed with the antigen peptide by using $3 \mu\text{g}$ peptide (ml antiserum) $^{-1}$, pre-absorption eliminated reactivity, both in immunoprecipitation of *in vitro*-translated E5 labelled with [^{35}S]methionine (Fig. 1e) and in immunostaining experiments (Fig. 1f). E5 expression in the differentiated layers of the papillomas was often accompanied by expression of the proliferation antigen Ki67 (Fig. 3g), detected by mAb MIB-1 (Dako). Expression of Ki67 confirmed the transformed nature of these cells: in normal epithelia, cells cease to proliferate once they leave the basal layer.

To analyse expression of MHC I in papillomas, we incubated sample sections with mAb IL-A88, which detects the heavy chain of bovine MHC I (Toye *et al.*, 1990). In normal epithelium, there was strong staining of the capillaries and

stromal cells; the epithelium was stained throughout most of its thickness (Fig. 2a) and staining on the surface of the cells was particularly clear in cells of the basal and suprabasal layers (Fig. 2b). There was no staining without primary antibody (data not shown). In sections of papillomas, MHC I could be detected in stroma and capillaries (Fig. 3e) and on the surface of some epithelial cells, as in normal tissue, but not in cells expressing E5 (Figs 2d and e, and 3a and b), independent of whether the cells were in the deeper layers (Fig. 3a and b), the suprabasal, transit and lower spinous layers (Fig. 2d and e, boxed area 1) or the more superficial layers (Figs 2d and e, and 3d and e). These results suggested that expression of E5 is incompatible with expression of MHC I.

The E7 proteins of HPV-16 and -11 have been implicated, respectively, in the downregulation of MHC I either through inhibition of the transcriptional promoter of the MHC I heavy chain (Georgopoulos *et al.*, 2000) or indirectly through inhibition of TAP, the transporter associated with peptide (Vambutas *et al.*, 2001). To ensure that the absence of MHC I in bovine papillomas was due to E5 and not to E7, we stained papilloma sections with rabbit antisera 11547 and 11823, which were raised against a β -galactosidase-E7 fusion protein (Anderson *et al.*, 1997). Similar results were obtained with both antisera; only results that were obtained with antiserum 11547 are shown. E5 and E7 are co-expressed in the same cells (Anderson *et al.*, 1997) and, accordingly, in



this study, cells that expressed either E5 or E7 alone were seldom detected. Nevertheless, in cells that expressed E5 and did not express E7, or expressed it at levels below detection, there was little or no MHC I (Fig. 3a–c). Conversely, cells that expressed E7, but not E5, still had detectable MHC I (Fig. 3d–f and h–j). Thus, it appears that expression of E7 is not responsible for downregulation of MHC I.

Although the lack of MHC I in the uppermost layers of the papillomas (Figs 2e and 3e) was consistent with the differentiated state of the cells, its absence in the basal (Fig. 3b) and the immediate suprabasal (transit and lower spinous) (Fig. 2e, boxed area 1) layers could not be attributed to cell differentiation, as MHC I was present in these areas of normal mucosa (Fig. 2a). Furthermore, MHC

Fig. 3. Expression of E5, E7, MHC I and Ki67 in BPV-4-induced papillomas. Representative papilloma sections stained with anti-E5 antiserum 274 (1:2000; a, d and h), anti-bovine MHC I heavy chain mAb IL-A88 (1:200; b, e and i) anti-E7 antiserum 11547 (1:250; c, f and j) or with anti-Ki67 mAb MIB-1 (1:200; g). (a) Papilloma 1803, showing expression of E5 in the basal layers. (b) Serial section of papilloma 1803, showing lack of expression of MHC I in cells expressing E5. (c) Serial section of papilloma 1803, showing lack of expression of E7 in the basal layers. (d) Papilloma 1810, showing E5 expression in differentiated keratinocytes, but not in basal cells. (e) Serial section of papilloma 1810, showing expression of MHC I in basal cells. (f) Serial section of papilloma 1810, showing expression of E7 in basal cells and co-expression with E5 in differentiated keratinocytes. The boxed area in (d) and the corresponding areas in (e) and (f) are shown at higher magnification in (h–j), respectively. (g) Serial section of papilloma 1810, showing expression of Ki67 in basal cells and in E5-expressing differentiated keratinocytes. Magnification, $\times 40$ (a–c, h–j); $\times 20$ (d–g).

I was absent in similar areas of papillomas where E5 was not expressed (Fig. 2d and e, boxed area 2).

We conclude that E5 inhibits the expression of MHC I in BPV-induced papillomas, corroborating and validating our observations on downregulation of MHC I by E5 *in vitro*.

HPV-16 and other high-risk HPV types induce cervical intraepithelial neoplasia (CIN), the precursor lesion of cervical cancer (zur Hausen, 2002). MHC I downregulation has been observed in CIN, but E5 expression was not investigated (Bontkes *et al.*, 1998). Given that HPV-16 E5 downregulates MHC I (Ashrafi *et al.*, 2004) and that HPV-16 E5 can be found in CIN samples (Chang *et al.*, 2001), it can be speculated that E5 is also responsible for MHC I downregulation in CIN.

It remains to be seen whether the E5-induced downregulation of MHC I leads to evasion of the host immune response, thus allowing the virus to establish infection and allowing the infection to persist.

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Appendix (d)

E5 Protein of Human Papillomavirus Type 16 Selectively Downregulates Surface HLA Class I

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Papillomaviruses have evolved mechanisms that result in escape from host immune surveillance. The E5 protein is expressed early in papillomavirus infection in the deep layers of the infected epithelium. It is localized to the Golgi apparatus (GA) and endoplasmic reticulum. The E5 protein of bovine papillomavirus (BPV) impairs the synthesis and stability of major histocompatibility (MHC) class I complexes and prevents their transport to the cell surface due to retention in the GA. Here we show that human papillomavirus type 16 (HPV-16) E5 also causes the retention of MHC (HLA) class I complexes in the GA and impedes their transport to the cell surface, which is rescued by treatment with interferon. Unlike BPV E5, HPV-16 E5 does not affect the synthesis of HLA class I heavy chains or the expression of the transporter associated with antigen processing TAP. These results show that downregulation of surface MHC class I molecules is common to both BPV and HPV E5 proteins. Moreover, we determined that HPV-16 E5 downregulates surface expression of HLA-A and HLA-B, which present viral peptides to MHC class I-restricted cytotoxic T lymphocytes (CTLs), but not the natural killer (NK) cell inhibitory ligands HLA-C and HLA-E. Selective downregulation of cell surface HLA class I molecules may allow the virus to establish infection by avoiding immune clearance of virus-infected cells by both CTLs and NK cells.

Key words: human papillomaviruses; E5 oncoprotein; HLA class I; HLA-A/B; HLA-C/E

Papillomaviruses (PVs) are small DNA tumor viruses that infect the epithelia of humans and animals, causing benign hyperproliferative lesions. In most cases, PV infections are cleared after several months following activation of the host immune system against viral antigen.¹ However, occasionally the lesions do not regress and can progress to cancer. Certain PVs are more commonly associated with malignancy, including the human PV (HPV) types 16 and 18, high-risk viruses for the development of cervical cancer in women,² and bovine PV (BPV) type 4, associated with carcinomas of the alimentary canal in cattle.³ Persistent viral infection is required for neoplastic progression and failure of virus clearance is attributed to a poor immunologic response.

The PV genome encodes 3 transforming proteins, E5, E6 and E7. E5 is a small hydrophobic protein ranging in size from 42 amino acid residues in BPV-4 to 83 amino acid residues in HPV-16. E6 and E7 are the main transforming proteins of HPV.^{4,5} E5 is the major transforming protein of BPV and plays a lesser role in transformation by HPV.⁶ While E6 and E7 are expressed throughout the course of the disease and are necessary for the maintenance of a transformed phenotype, E5 is expressed during the early stages of infection and its expression is often, but not always, extinguished as the lesion progresses toward malignancy.⁶ These characteristics point to a role of E5 in establishment of PV infection and the initiation of cell transformation.

The E5 protein is localized in the Golgi apparatus (GA), endoplasmic reticulum and occasionally the plasma membrane of the host cell. Its localization in the endomembrane compartments, where it interacts with the vacuolar ATPase 16k ductin/subunit c,^{7–9} is deemed responsible for the lack of acidification of the GA and endolysosomes and the consequent impaired functions of these organelles.^{10,11}

We have shown that one of the outcomes of BPV E5 expression in primary cells is the retention of major histocompatibility (MHC) class I complexes in the GA and the inhibition of their transport to the cell surface.^{12,13} Furthermore, BPV E5 inhibits both transcrip-

tion of the MHC class I heavy chain gene and affects the stability of the heavy chain protein.¹² In this study, we show that HPV-16 E5 also prevents the transport of MHC (HLA) class I complexes to the cell surface due to retention in the GA. Moreover, we show that HPV-16 E5 selectively downregulates HLA-A and HLA-B molecules on the cell surface but does not affect the transport of HLA-C and HLA-E. These studies identify a potential novel mechanism by which PV-infected cells may avoid clearance by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, aiding in the establishment and persistence of PV infection.

Material and methods

HPV-16 E5 expression constructs

The E5 ORF was cloned into 3 expression plasmids: pcDNA3 (Invitrogen, Glasgow, U.K.), under the transcriptional control of the universal cytomegalovirus (CMV) immediate early promoter (pc-16E5); pL2, under control of the Epstein-Barr virus (EBV) ED-L2 promoter, active only in epithelial cells¹⁴ (pL2-16E5); and the retrovirus expression plasmid pLZRSpBMLZ (Clontech, Palo Alto, CA). Amphotropic retrovirus expressing HPV-16 E5 (RT-16E5) was generated by transient transfection of the packaging cell line Phoenix¹⁵ as previously described.¹²

Establishment of HPV E5-expressing cell lines

The immortalized human keratinocyte HaCaT cell line was grown in Dulbecco's modified eagle medium (DMEM) without CaCl₂ (Invitrogen), supplemented with 1 mM sodium pyruvate, 2 mM glutamine and 10% fetal calf serum (FCS) at 37°C in 5% CO₂. Primary bovine PalF cells¹² and NIH 3T3 cell lines were grown in DMEM, 10% FCS at 37°C in 5% CO₂.

HaCaT cells were stably transfected with 4 µg of pcDNA, pL2, pc-16E5, or pL2-16E5 per 1 × 10⁶ cells using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. Following transfection, the cells were selected in DMEM containing 500 µg/ml G418 (Invitrogen) for 21 days. After this time, G418-resistant colonies were marked, individually picked and expanded into clonal cell lines for analysis. The retrovirus RT-16E5, or its empty counterpart (generated using wild-type pLZRSpBMLZ plasmid), was used to infect primary PalF cells as described¹² and the cells were analyzed 2 days later.

NIH3T3 cells expressing HPV-6b E5 or HPV-16 E5 under the control of the mouse moloney leukemia virus (MLLV) LTR in pZip-neo were a kind gift from Dr. Show-Li Chen (National Defense Medical Center, Taipei, Taiwan) and Professor Richard Schlegel (Georgetown University, Washington, DC), respectively.

Quantitative RT-PCR

Total RNA was isolated from HaCaT cells using the RNeasy Mini Kit (Qiagen, Crawley, U.K.), and residual DNA was removed

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by DNase I treatment (Invitrogen). Real-time RT-PCR for HPV-16 E5 and β -actin mRNA was carried out using the Taqman EZ RT-PCR kit (Applied Biosystems, Foster City, CA). Each reaction was performed in triplicate using 100 ng of RNA. Oligonucleotide primers, designed using Primer Express (v1.7, Perkin-Elmer, Oak Brook, IL), were as follows: 16E5wt F 5'-TGACAAATCTTG-ATACTGCATCCA-3'; 16E5wt R 5'-CTGCTGTTATCCACA-ATAGTAATACCAATA-3'; and a FAM/TAMRA probe 5'-AAC-ATTACTGGCGTGCTTTTGGCTTTGCT-3'. Primers and probe sequences for β -actin quantitation were purchased from Applied Biosystems. PCR reactions were performed using an ABI Prism 7700 Sequencer. Standard curves were generated using 10-fold serial dilutions of each template DNA, which were used to quantitate the relative levels of E5 and β -actin mRNA. E5 mRNA levels were normalized according to the β -actin controls.

FACS analysis of MHC class I expression

HaCaT, PalF and NIH3T3 cells were grown in T175 cm² flasks until subconfluent. After removal of the medium, the cells were washed once with PBS, then detached from the flask with trypsin/EDTA and pelleted at 200g for 5 min at room temperature (RT). The cell pellet was resuspended in DMEM, 10% FCS, and incubated for 1 hr at 37°C to allow surface antigens to be reexpressed. The cells were then washed and resuspended in PBS/1% bovine serum albumin (BSA; PBS-B) at a concentration of 10⁷ cells/ml. For the detection of surface MHC class I molecules, 100 μ l of cells were aliquoted and incubated for 1 hr at 4°C with an equal volume of monoclonal antibody (mAb) as follows: pan antihuman MHC class I W6/32 (1:100; Serotec, Oxford, UK), anti-bovine MHC class I IL-A19 (1:1,000),¹⁶ anti-mouse H-2L^d CL9011-A (1:50; Cedarlane Laboratories, Newcastle, UK), or anti-HLA-C/E DT9 (1:50; a kind gift from Dr. Veronique Braud, Centre National de la Recherche Scientifique, Sophia Antipolis, France) for 30 min at 4°C. Following 3 washes in PBS-B, cells were incubated with 1:100 dilution of anti-mouse IgG-FITC (Sigma, St. Louis, MO) for 30 min at 4°C in the dark. The cells were then washed as above, resuspended in 500 μ l PBS-B and analyzed by flow cytometry. If the flow cytometry analysis was not performed immediately, the cells were resuspended in 500 μ l of 3% paraformaldehyde in PBS and kept at 4°C. A mouse monoclonal antibody against HPV-16 E2 (TVG261; a kind gift of Dr. M. Hibma) was used as negative control (1:50).

For the detection of intracellular MHC class I, the cells were first permeabilized with 0.5% saponin in PBS-B for 30 min at RT. Following a wash in PBS-B, the cells were then incubated with primary antibody as described above. All samples were examined in a Beckman Coulter EPICS Elite analyzer equipped with an ion argon laser with 15 mV of excitation at 488 nm. Data were analyzed using Expo 2 software.

Immunofluorescence detection of MHC class I and GA

In all experiments, HaCaT cells (1×10^4) were aliquoted into 24-well plates containing coverslips and grown overnight. After removal of the medium, cells were washed twice with PBS and fixed in 1.85% formaldehyde in PBS containing 2% sucrose for 10 min at RT. After fixation, cells were washed twice and incubated in permeabilizing solution (0.5% NP-40, 10% sucrose in PBS) for 10 min at RT and then washed as above.

For detection of MHC class I, the fixed and permeabilized cells were incubated with 1:50 dilution of W6/32 antibody for 1 hr at RT. Following 2 further washes, the cells were incubated with 1:500 dilution of anti-mouse IgG-FITC (Sigma) for 1 hr at 4°C in the dark. For visualization of the GA, the cells were incubated with mAb 4A3 (1:200) recognizing golgin GM130, an integral GA protein,¹⁷ for 1 hr at RT. Following 2 washes as above, the cells were incubated with 1:1,000 dilution of anti-mouse IgG-TRITC (Sigma) for 1 hr at 4°C in the dark.

To analyze the localization of MHC class I in E5-expressing cells, control HaCaT cells (pcDNA and pL2) and E5-expressing cells (pc-16E5 and pL2-16E5) were incubated with mAb 4A3,

washed as described above, then incubated with anti-mouse IgG-TRITC (1:1,000) and FITC-conjugated W6/32 (1:10; Sigma).

In all cases, the cells were washed 3 times after incubation with secondary antibody, and the coverslips were mounted onto slides using Citifluor. Images were captured using a Leica TCS SP2 true confocal scanner microscope (Leica Microsystems, Heidelberg, Germany) and a wavelength of 488 nm (MHC class I) or 543 nm (GA). The merge between the FITC and TRITC fluorescent signals was achieved using the Leica TCS SP2 accompanying software.

Immunoblotting detection of MHC class I and TAP

HaCaT cells were removed from the flasks by trypsinization, washed with PBS, then lysed by sonication in lysis buffer (100 mM Tris HCl, pH 7.5, 2% SDS, 20% glycerol) and insoluble material was removed by centrifugation at 20,000g for 10 min at 4°C; 10 μ g of lysate were electrophoresed in 4–12% NuPAGE gels (Invitrogen), and proteins were transferred to nitrocellulose membrane using a semidry blotting apparatus at 20 V/150 A for 1 hr. The membranes were blocked in 5% milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST) for 1 hr at RT.

For detection of MHC class I, the membranes were incubated with the following mAb: HC10, specific for HLA class I heavy chain (1:50; a kind gift from Dr. Stephen Man, Cardiff University, Cardiff, U.K.), MEM-E/02 specific for human HLA-E (1:50; Serotec), or AB-1 (1:20,000; Oncogene Research Products, Nottingham, UK) specific for actin. For detection of the transporter associated with antigen processing TAP, the membranes were incubated in 1:1,000 dilution of rabbit anti-TAP-1 antibody (Chemicon, Hampshire, UK). After repeated washing with TBST, the membranes were incubated with either 1:20,000 dilution of anti-mouse Ig-HRP (Amersham Pharmacia Biotech, Bucks, UK) for HC10 and MEM-E/02, 1:5,000 dilution of anti-mouse IgM-HRP (Oncogene Calbiochem-Novabiochem, Nottingham, UK) for AB-1, or 1:5,000 dilution anti-rabbit IgG-HRP (Sigma) in 5% milk/TBST for 1 hr at RT. The membranes were washed 3 times with TBST and bound antibody was detected by enhanced chemoluminescence (ECL; Amersham Pharmacia Biotech).

Treatment of cells with interferon

One million parental HaCaT cells, control cells expressing pcDNA3 or pL2 and cells expressing HPV-16 E5 were seeded in T175 cm² tissue culture flasks. The following day, the medium was replaced with fresh medium with or without 500 U/ml IFN- β (Sigma). After 48 hr, the cells were harvested for detection of MHC class I by immunofluorescence or flow cytometry analysis as described above.

Results

Detection of E5 expression in cell lines

As E5 is expressed at very low levels in cells and there are no reliable antibodies against the protein, it is very difficult to detect its expression by immunoblotting.¹⁸ Therefore, we confirmed instead that the E5 ORF was being transcribed using quantitative RT-PCR. Ten clones from each HaCaT cell transfection with pcDNA, pL2, pc-16E5, or pL2-16E5 were picked and expanded into cell lines for analysis. RNA was isolated and the relative level of E5 mRNA expression was determined by comparison to β -actin mRNA. The results of 6 clones expressing HPV-16 E5 (3 as pc-16E5 and 3 as pL2-16E5) are shown in Figure 1. Although low (4 orders of magnitude less than that of β -actin), all of the cell lines expressed E5 mRNA. The amount of E5 mRNA was comparable among each cell line, ranging from approximately 0.01 to 0.02 pg per 100 ng of RNA. The levels of E5 mRNA in HaCaT keratinocytes are approximately 100-fold lower than those in W12 cells (derived from cervical intraepithelial neoplasia),¹⁹ which express E5 from the resident multicopy episomal HPV-16 genome (data not shown). This observation therefore excludes the possibility that any effect seen in HaCaT cells is due to E5 overexpression.

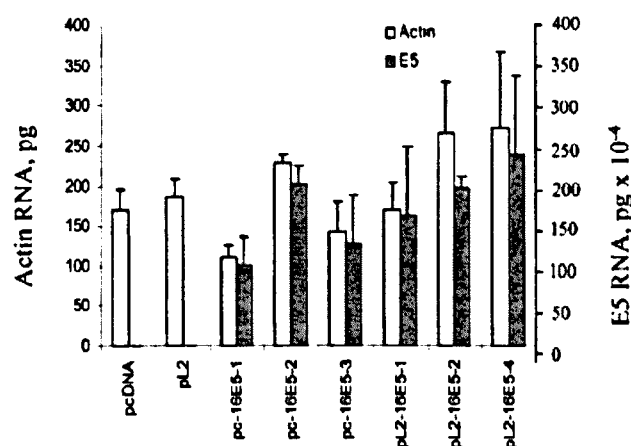


FIGURE 1 – Expression of E5 RNA in transfected cell lines. HaCaT cells were transfected with pcDNA3 (pcDNA), pcDNA3 expressing the HPV-16 E5 ORF (pc-16E5), pL2, or pL2 expressing the E5 ORF (pL2-16E5). Quantitative RT-PCR was used to determine the relative amount of HPV-16 E5 and β -actin mRNA in 3 of each cell line. The results of 6 representative cell lines are shown. Relative RNA values are expressed as the mean of 3 independent experiments \pm standard deviation.

Downregulation of surface HLA class I in cells expressing HPV E5

Given that BPV E5 can inhibit transport of MHC class I molecules to the cell surface,^{12,13} we investigated whether HPV-16 E5 could likewise downregulate surface HLA class I. Using FACS analysis, we determined the levels of HLA class I in HaCaT cell lines expressing HPV-16 E5 under the control of the CMV promoter (pc-16E5) or expressing E5 under the control of the epithelial-specific EBV promoter (pL2-16E5). HaCaT cells harboring empty plasmids expressed similar levels of HLA class I as parental HaCaT cells, with approximately twice as much total (surface plus intracellular) HLA class I than surface alone (Fig. 2a). In contrast, all of the pc-16E5 and pL2-16E5 HaCaT cell lines analyzed had reduced levels of surface HLA class I, approximately half that of the control or parental cells, whereas the level of total HLA class I remained constant (Fig. 2a). This effect was highly reproducible and specific as no signal above background (secondary antibody only) was detected when an anti-HPV-16 E2 antibody was used (Fig. 2c). Furthermore, we showed a marked reduction of surface MHC class I in NIH 3T3 mouse fibroblasts expressing HPV-6 E5 or HPV-16 E5 under the MMLV LTR and in primary bovine PalF cells acutely infected with recombinant retrovirus expressing HPV-16 E5 (RV-16E5; Fig. 2b). These results show that downregulation of MHC class I is stimulated by E5 proteins encoded by both BPV and HPV, including low- (HPV-6) and high-risk (HPV-16) viruses. Moreover, stimulation of MHC class I downregulation in primary cells (PalF) shows that this effect is not due to the immortalized phenotype of the HaCaT keratinocytes.

HLA class I is retained in GA in E5-expressing epithelial cells

To ascertain the intracellular localization of HLA class I, E5-expressing HaCaT cell lines, parental and control cell lines were costained with the antibodies W6/32 (pan MHC class I) and 4A3 (antigolgin). In the control cells, HLA class I was expressed both on the cell surface and in the GA (Fig. 3a). Identical staining patterns were observed in parental HaCaT cells (data not shown). In contrast, in HPV-16 E5-expressing cells, HLA class I was detected almost exclusively in the GA (Fig. 3b). These results show that HPV-16 E5 prevents the HLA class I complex from reaching the cell surface and retains it in the GA.

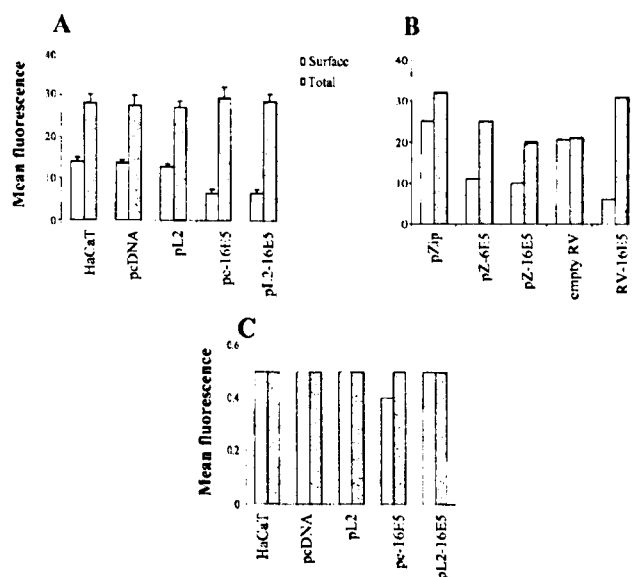


FIGURE 2 – HPV E5 downregulates surface HLA class I. (a) Parental HaCaT cells (3 lines), cells harboring empty vectors (pcDNA3, 3 lines; pL2, 4 lines) or expressing HPV-16 E5 (pc-16E5, 4 lines; pL2-16E5, 3 lines) were analyzed for expression of total and surface HLA class I by FACS with mAb W6/32. The average mean fluorescence for each expression vector was calculated from the flow cytometric analyses. A background of 0.4 (the reading of cells stained with no primary antibody and only secondary antibody) was subtracted in all cases. Standard deviation (\pm) is shown. (b) FACS analysis of surface and total MHC class I in NIH 3T3 cells carrying empty vector (pZip), expressing HPV-6b E5 (pZ-6E5) or HPV-16 E5 (pZ-16E5), and in PalF cells carrying empty retrovirus (empty RV) or expressing HPV-16 E5 (RV-16E5). One cell line of each was analyzed. (c) FACS analysis with an unrelated antibody (anti HPV-16 E2) showing no reaction above background.

Expression of HLA class I heavy chain is not inhibited by HPV-16 E5

The results above suggested that, in contrast to BPV E5, HPV-16 E5 did not have any effect on the overall levels of the HLA class I heavy chain. To confirm this observation, we determined the relative levels of HLA class I in the control and E5-expressing HaCaT cell lines using mAb HC10, specific for the human HLA class I heavy chain.²⁰ Although the levels of HLA class I heavy chain were slightly lower in the pL2 and pL2-16E5 cell lines than in the pcDNA and pc-16E5 cell lines, there were no significant differences between the cells expressing HPV-16 E5 and their respective control cells (Fig. 4), confirming that HPV-16 E5 does not downregulate expression of the HLA class I heavy chain.

E5 has no effect on TAP expression

Transport of class I complexes to the cell surface is prevented if the transporter associated with antigen processing (TAP) is malfunctioning.²¹ Therefore, it was important to establish whether E5 was affecting HLA class I transport by inhibiting TAP expression, as reported for HPV-11 E7.²² TAP-1 protein was investigated in control and E5-expressing HaCaT cell lines by immunoblotting. There appeared to be a lower level of TAP-1 in the pL2 cells but there was no significant reduction of TAP-1 in cells expressing E5 (Fig. 4), indicating that downregulation of surface HLA class I is not due to an ability of E5 to downregulate TAP-1. An effect on the functionality of TAP cannot be ruled out, however.

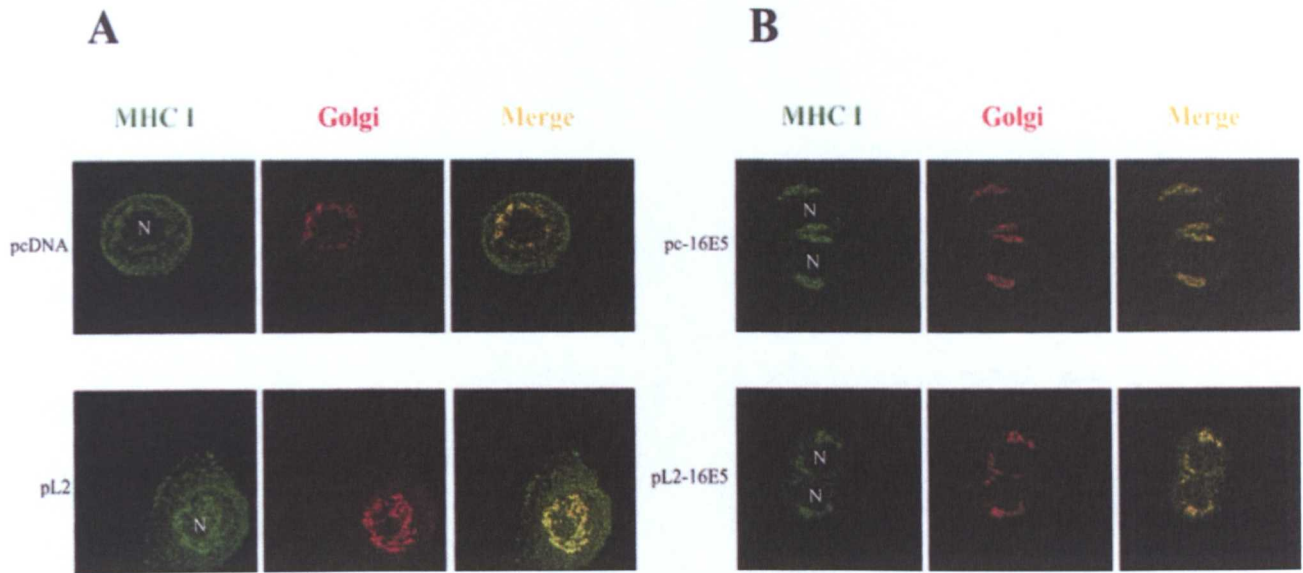


FIGURE 3 – HLA class I is retained in the Golgi apparatus in HPV-16 E5-expressing cells. HaCaT cells carrying empty vectors or expressing E5 (at least 3 lines of each) were stained with mAb W6/32 (anti-HLA class I) and mAb 4A3 (antigolgin GM130) and analyzed using confocal microscopy. N, nucleus. Representative cells are shown. (a) Control HaCaT cells carrying either pcDNA or pL2 empty vector. (b) HeCaT cells expressing HPV-16 E5 in either pcDNA (pc-16E5) or pL2 (pL2-16E5).

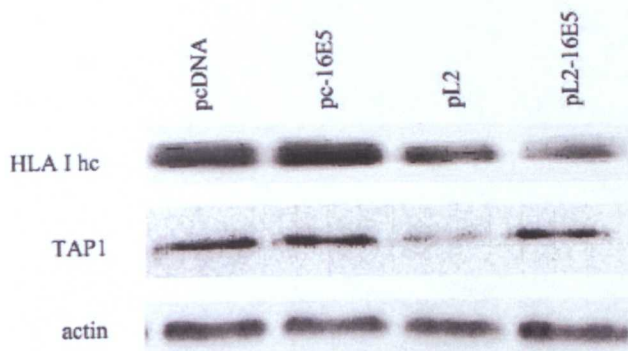


FIGURE 4 – HPV-16 E5 does not inhibit expression of HLA heavy chain (hc) or TAP. Equal amounts (10 μ g) of protein lysates from one line each of HaCaT cells carrying empty vectors (pcDNA, pL2) or expressing HPV-16 E5 (pc-16E5, pL2-16E5) were analyzed by immunoblotting with mAb HC10 (anti-hc), anti-TAP-1 antiserum, or mAb AB-1 (antiactin).

Treatment of E5 cells with interferon rescues HLA class I traffic to cell surface

Interferon- β (IFN- β) increases transcription from the MHC class I heavy chain gene promoter²³ leading to higher expression of heavy chain. To determine if increased synthesis of heavy chain led to an increase in transport of HLA class I complexes to the cell surface, we treated HaCaT parental, control and E5-expressing cells with IFN- β and analyzed HLA class I expression and localization by FACS and immunofluorescence. Treatment with IFN- β increased the total amount of HLA class I approximately 2-fold in all of the cell lines tested (Fig. 5a compared with Fig. 2a). Moreover, we found an approximately 2-fold increase in surface HLA class I in the parental and control cells and an approximately 4-fold increase in the E5-expressing cells (Fig. 5a compared with Fig. 2a). This resulted in all of the cell lines, including those expressing HPV-16 E5, as having similar levels of surface HLA class I. IFN- β treatment did not affect expression of E5 (data not shown) and therefore the observed increase in HLA class I levels

cannot be attributed to changes in E5 expression. Unlike in BPV E5-expressing cells,¹³ IFN- β treatment appeared to overcome the block exerted by E5 on HLA class I transport. To confirm this observation, HaCaT cells carrying empty vector or expressing HPV-16 E5, untreated or treated with IFN, were incubated with mAb W6/32 and analyzed for HLA class I localization. In the untreated E5-expressing cells, HLA class I was mostly detected in the GA as before (Fig. 5b). In contrast, HLA class I was detected on the surface of E5-expressing cells treated with IFN. These experiments show that HPV-16 E5-expressing cells are responsive to IFN and that the E5-induced HLA class I transport inhibition is reversible by IFN.

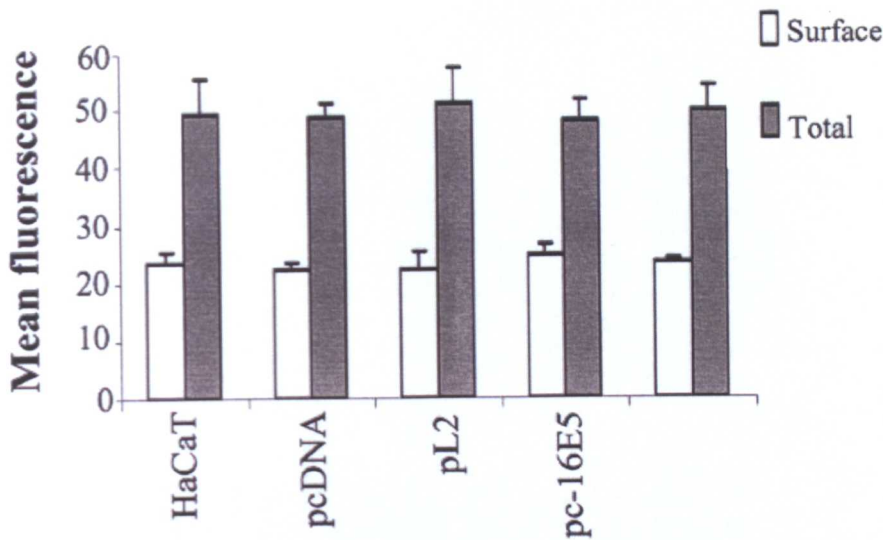
HLA-C/E expression is not inhibited by HPV-16 E5

While MHC class I molecules HLA-A and -B are the main presenters of antigenic peptides to CTLs, HLA-C and nonclassical MHC molecules, such as HLA-E, inhibit NK cell-mediated lysis by interacting with inhibitory NK receptors.^{24–26} To determine whether HPV-16 E5 could selectively downregulate HLA class I molecules, we determined the levels of HLA-C/E in parental, control and E5-expressing HaCaT cell lines. Cells were stained with mAb DT9 that recognizes both HLA-C and -E and were analyzed by flow cytometry for surface and total HLA-C/E. Although the shift in forward fluorescence was small (Fig. 6a and b), in agreement with the observation that human fibroblasts have little HLA-E,²⁷ it was consistently higher than background (secondary antibody only; Fig. 6b) and higher than the readings obtained with an unrelated antibody (Fig. 2c). Importantly, there were no significant differences between the control and E5-expressing cells (Fig. 6a).

In addition, we determined the cellular localization of HLA-C/E in the E5-expressing cells by immunofluorescence using mAb DT9. There were no differences between the staining patterns of HLA-C/E in the control cells and in the E5-expressing cells (Fig. 6c); therefore, we conclude that expression of E5 does not lead to any appreciable decrease in surface HLA-C/E, in agreement with the FACS data.

Finally, we determined the levels of total HLA-E by immunoblotting with mAb MEM-E/02, specific for HLA-E. Similarly, we did not detect any significant differences between HLA-E levels in

A



B

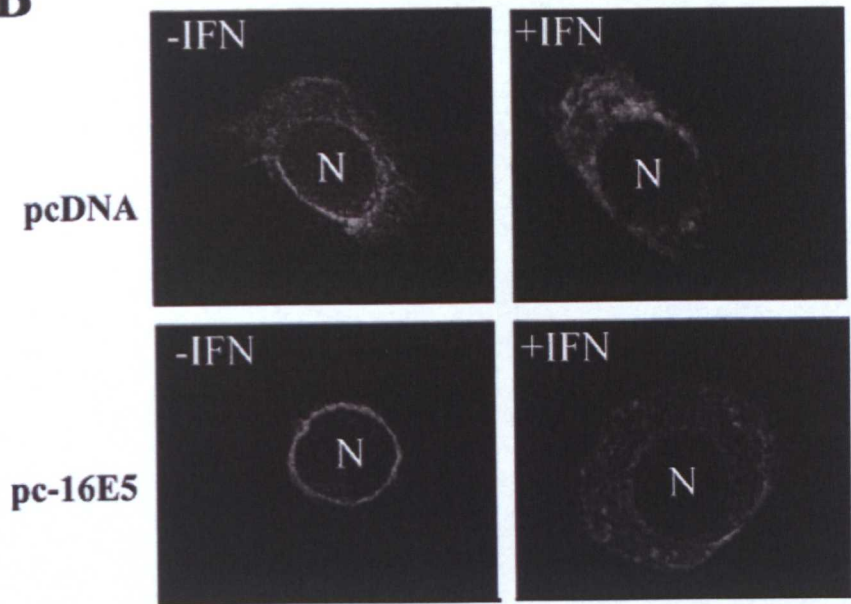


FIGURE 5 – IFN treatment rescues transport of HLA class I to the cell surface. (a) Parental HaCaT cells (3 lines), cells carrying empty vectors (pcDNA and pL2, 3 lines each), or cells expressing HPV-16 E5 (pc-16E5, pL2-1E5, 3 lines each) were treated with 500 U/ml IFN- β for 48 hr and analyzed for expression of total and surface HLA class I by FACS analysis with mAb W6/32. The average mean fluorescence for each expression vector was calculated from the flow cytometric analyses. A background of 0.4 (the reading of cells stained with no primary antibody and only secondary antibody) was subtracted in all cases. Standard deviation (\pm) is shown. (b) Immunofluorescence detection of HLA class I with MAb W6/32 in at least 3 cell lines carrying empty vector (pcDNA) or expressing HPV-16 E5 (pc-16E5), untreated or treated with IFN- β as in (a). N, nucleus. Representative lines are shown.

the control cells and the E5-expressing cells (Fig. 6d). The ease of HLA-C/E detection by immunofluorescence and immunoblotting compared with flow cytometry is likely attributable to the different affinities of the 2 antibodies for HLA (mAb DT9 and MEM-E/02) and to the greater sensitivity of mAb DT9 in immunofluorescence. These results confirm that HPV-16 E5 downregulates the surface expression of the classical HLA class I molecules HLA-A and -B, but not HLA-C or -E. We are unable to discriminate between HLA-C and HLA-E as mAb DT9 recognizes both molecules, and mAb MEM-E/02, specific for HLA-E, does not function in flow cytometry or immunofluorescence.

Discussion

Progression from acute HPV infection to malignancy requires persistence of virus, which in turn appears to depend on several

factors, including the genetic background of the host,^{28,29} environmental cofactors³⁰ and the ability of the virus to avoid immune clearance.³¹ The immune system plays a decisive role in determining the clinical outcome of HPV disease, as demonstrated by the increased persistence and enhanced neoplastic progression of HPV infections in hosts with cell-mediated immune deficiencies.^{32,33} However, even in immunocompetent individuals, HPVs persist for a significant period of time before activation of the host immune system. This lack of recognition suggests the host immune system is unaware of, or disabled by, HPV infection. HPVs can subvert the immune response indirectly via the nature of the virus life cycle³⁴ and by direct interference with the host antiviral immune mechanisms, including the IFN response and MHC class I antigen presentation to CTLs.^{1,35}

MHC class I (HLA class I in humans) plays a pivotal role in the eradication of virally infected and transformed cells. The impor-

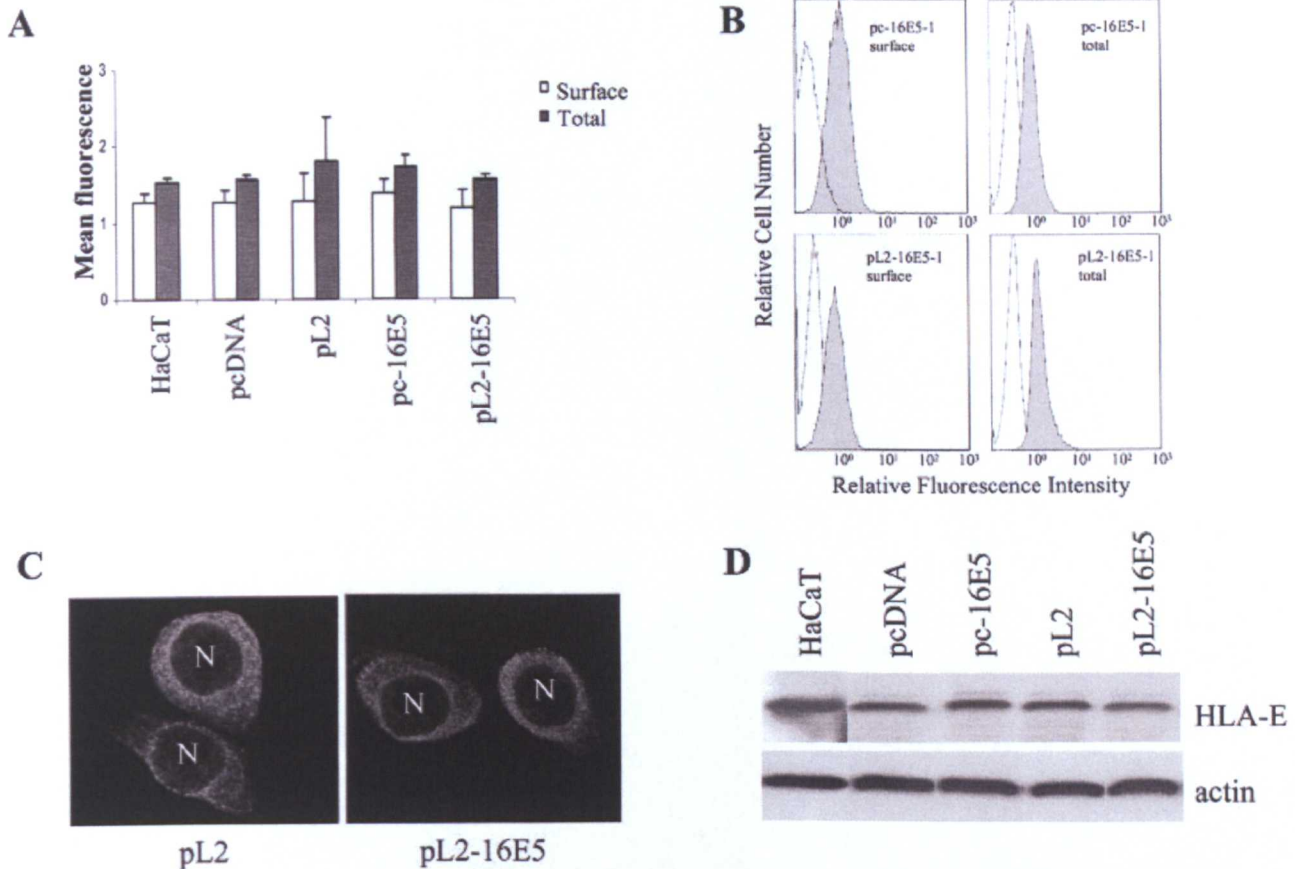


FIGURE 6 – HPV-16 E5 does not downregulate HLA-C/E. (a) FACS analysis of surface and total HLA-C/E with mAb DT9 in parental HaCaT cells (3 cell lines), cells carrying empty vectors (pcDNA and pL2, 3 lines for each vector) or expressing HPV-16 E5 (3 lines of pc-16E5 and 4 of pL2-16E5). The average mean fluorescence for each expression vector was calculated from the flow cytometric analyses (example in b). A background of 0.4 (the reading of cells stained with no primary antibody and only secondary antibody) was subtracted in all cases. Standard deviation (\pm) is shown. (b) Representative FACS profiles of pc-16E5 and pL2-16E5 cells (one line of each). Dotted line and open histogram: forward fluorescence with secondary antibody only; solid histogram: forward fluorescence with primary and secondary antibody. (c) Immunofluorescence detection of HLA-C/E with mAb DT9 in 2 lines each of cells carrying empty vector (pL2) or expressing HPV-16 E5 (pL2-16E5). N, nucleus. (d) Detection of HLA-E heavy chain by immunoblotting. Protein lysates from 3 lines each of parental HaCaT cells, cells carrying empty vector (pcDNA3 or pL2) or expressing E5 (pc-16E5 or pL2-16E5) were analyzed by immunoblotting with mAb MEM-E/02 (anti-HLA-E) or mAb AB-1 (antiactin). Representative lines are shown.

tance of MHC class I in virus clearance is highlighted by the acquisition of numerous mechanisms of interference with the MHC class I pathway by many viruses.³⁶ Independently of the molecular nature of these mechanisms, the outcome is failure of the infected cells to present viral peptides to effector CTLs effectively, resulting in avoidance of detection and destruction.

We have recently shown that both BPV-4 and BPV-1 interfere with the MHC class I pathway through the retention of MHC class I complexes in the GA by the oncoprotein E5.^{1,12,13} Here we show that these properties are not a peculiarity of BPV E5 but are shared by E5 proteins of mucosal HPV, including E5 encoded by the low-risk HPV type 6, the etiologic agent of genital warts and HPV-16, the papillomavirus most frequently associated with cervical carcinoma. Since our initial observations were published, it has also been reported that HPV2a E5 can inhibit HLA class I transport to the cell surface.³⁷ Therefore, downregulation of surface MHC class I appears to be a property of many, if not all, papillomavirus E5 proteins.

We found that HPV-16 E5 promotes the retention of HLA class I in the GA, a salient characteristic of cells expressing BPV-4 E5. It is established that E5 proteins bind 16k subunit c, a component of the V_0 sector of the H^+ V-ATPase,⁷⁻⁹ and that a possible outcome of this interaction is the inhibition of acidification of the

GA and endosomes.^{10,11} The retention of MHC class I in the GA by BPV-4 E5 is due at least in part to the impeded acidification of the organelle, as retention in the GA and downregulation of surface MHC class I are also caused by monensin, an inhibitor of V-ATPase and GA acidification.¹³ The same mechanism may underpin the retention of HLA class I in the GA by HPV-16 E5. However, given the selective downregulation of HLA types by HPV-16 E5, the lack of GA acidification cannot be the whole explanation and other mechanisms must come into play. These points are currently under investigation.

Despite the similarities, there are also differences between the extent to which BPV-4 E5 and HPV-16 E5 interfere with the MHC class I pathway. BPV-4 E5 downregulates transcription of the MHC class I heavy chain gene, promotes degradation of the translated polypeptide and blocks the transport of the MHC class I complex to the cell surface.^{12,13} In contrast, HPV-16 E5 does not inhibit expression of the heavy chain, but reduces the transport of HLA class I to the cell surface without completely abolishing it. Furthermore, contrary to what we observed with BPV E5,¹³ inhibition of HLA class I transport by HPV-16 E5 is reversible by IFN treatment. The reason for this latter difference is not known but it can be speculated that the increased production of HLA class I heavy chain by IFN is sufficient to overcome the inhibitory effect

mediated by the low levels of HPV-16 E5. In contrast, as BPV E5 also inhibits transcription and promotes degradation of the MHC class I heavy chain,¹² IFN treatment is insufficient to restore MHC class I expression to normal levels. These data are also consistent with our hypothesis that there is a correlation between protein oncogenicity and immune evasion.³⁸ As discussed earlier, BPV E5 is a more effective transforming protein than HPV-16 E5⁶ and therefore would be predicted to have a greater effect on MHC class I downregulation (and other immune evasion mechanisms) than HPV-16 E5. In contrast, in oncogenic HPV infections, the 2 major transforming proteins E6 and E7 would complement the inhibitory effect of the lesser transforming protein E5 on the MHC class I pathway. HPV-16 E7 can repress the MHC class I heavy chain gene promoter,³⁹ thus likely replacing the inhibitory action of BPV on the same promoter, and in addition can bind directly to TAP, thus further contributing to the downregulation of HLA class I.²¹ Furthermore, both HPV E6 and E7 can inhibit the type I IFN pathway, thus preventing the IFN-mediated release of E5-induced blockage in HLA class I trafficking.^{40–42}

Although an efficient mechanism to avoid CTL-mediated immune clearance, the total absence of surface MHC class I renders cells more susceptible to NK cell attack. Human NK cells express multiple receptors that interact with HLA class I molecules, including killer cell immunoglobulin-like receptors (KIRs) that predominantly recognize classical HLA class I, including HLA-C, and the C type lectin superfamily of receptors that specifically interact with the nonclassical class I molecule HLA-E.^{24–26} Recognition of the class I molecules by their inhibitory receptors inhibits NK-mediated cell lysis, which would occur in the absence of HLA-C/E. Accordingly, certain viral proteins, including HIV Nef and the US3/UL40 proteins of CMV, have evolved to downregulate selectively HLA-A and -B, the main presenters of peptides to CTLs, but not HLA-C or -E,^{27,43,44} and are therefore capable of avoiding both CTL and NK cell killing.⁴⁵ We show here for the first time that papillomaviruses, in particular HPV-16, may also employ a similar immune evasion strategy via expression of E5. Our experiments show that neither synthesis nor transport to the cell surface of HLA-C/E is affected by E5 expression, leading to the conclusion that E5 selectively inhibits surface expression of HLA-A and HLA-B. NK cells of patients with HPV-induced anogenital lesions are incapable of specific killing HPV-16-infected cells,⁴⁶ although the mechanism by which this occurs is as yet unknown. Moreover, concordant with loss of MHC class I molecules that present viral peptides, there is a very low frequency

of HPV-specific HLA-A-restricted CTLs in patients infected with HPV-16, an order of magnitude lower than those found in other viral infections, including influenza A and EBV.⁴⁷ It is not yet known whether these observations are the consequence of E5 expression; however, experiments to establish the functional outcome of E5 expression on CTL and NK cell recognition of HPV-transfected cells are in progress.

Regardless, our results support the hypothesis that E5 plays a major role in immune evasion by HPV. To this end, it is interesting to note that HPV-16 E5 has also been reported to inhibit both Fas ligand- and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in HaCaT cells⁴⁸ and, consistent with its role in the alkalization of endosomes, prevents the endosomal breakdown of the invariant chain,⁴⁹ a chaperone important in the maturation of HLA class II, leading to inhibition of expression of surface HLA class II.⁴⁹ Therefore, E5 can disrupt several critical components of the cell-mediated immune response to viruses, which may contribute to the establishment and persistence of HPV infection.

It remains to be seen if E5 expression causes HLA class I downregulation also *in vivo*. This appears to be the case in bovine papillomas (data not shown). HLA class I downregulation has been observed in CIN⁵⁰ and in cervical carcinomas.⁵¹ However, the downregulation of HLA class I in cervical carcinomas, which often do not express E5, is common to other cancer types and therefore unlikely to be due to E5. No correlation was made between HLA class I downregulation and E5 expression in CIN, and this point warrants further investigation.

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Appendix (e)

Similarities and differences between the E5 oncoproteins of bovine papillomaviruses type 1 and type 4: Cytoskeleton, motility and invasiveness in E5-transformed bovine and mouse cells

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Abstract

Bovine papillomaviruses (BPVs) are oncogenic viruses. In cattle, BPV-1/2 is associated with urinary bladder cancer and BPV-4 with upper GI tract cancer. BPV E5 is a small hydrophobic protein localised in the endoplasmic reticulum (ER) and Golgi apparatus (GA). E5 is the major transforming protein of BPVs, capable of inducing cell transformation in cultured mouse fibroblasts and, in cooperation with E7, in primary bovine cells. E5-induced cell transformation is accompanied by activation of several cellular protein kinases, including growth factor receptors, and alkalisation of endosomes and GA. We have reported that BPV E5 causes swelling and fragmentation of the GA and extensive vacuolisation of the cytoplasm. We now show that E5 from both BPV-1 and BPV-4 disturbs the actin cytoskeleton and focal adhesions in transformed bovine cells, where these morphological and behavioural characteristics are accompanied by hyperphosphorylation of the cellular phosphotyrosine kinase c-src. Both BPV-1 and BPV-4 E5 increase the motility of transformed mouse cells, but only BPV-1 E5 causes transformed mouse cells to penetrate a matrigel matrix. BPV-1 transformed mouse cells, but not BPV-4 transformed mouse cells, have hyperphosphorylated c-src.
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Keywords: BPV; E5; Cell transformation; Cytoskeleton; Focal adhesions; Motility; Invasion; c-src

1. Introduction

Papillomaviruses (PVs) are oncogenic viruses, which induce benign hyperproliferative lesions of the cutaneous and mucous epithelia. PV infections are usually eradicated by a cell-mediated immune response directed against viral antigens. Occasionally, however, in a small but sizeable minority of cases the viral lesions do not regress and progress to cancer. High risk human papillomaviruses type 16 and 18 (HPV-16 and -18) are the main causative factor in the development of cancer of the cervix uteri (zur Hausen, 2002). Bovine papillomavirus type 1 and 2 (BPV-1, BPV-2) and BPV-4 are, respectively, involved in carcinogenesis of the urinary bladder and of the alimentary tract in cattle (Campo, 1997). BPV-1 also induces penile papillomas that can progress to cancer (Jarrett, 1985).

The BPV-1 genome encodes three transforming proteins, E5, E6 and E7, whereas BPV-4 does not possess an E6 gene (Jackson et al., 1991). For both viruses E5 plays a major role in cell transformation (Pennie et al., 1993; Venuti and Campo, 2002).

E5 is a small hydrophobic peptide of 44 amino acid residues in BPV-1 and 42 residues in BPV-4, localised in the endomembrane compartments of the endoplasmic reticulum (ER) and Golgi apparatus (GA) of the host cell. In warts and papillomas E5 is expressed in the cytoplasm of the basal and suprabasal layers and of the spinous and squamous layers. Expression is, however, discontinuous (Burnett et al., 1992; Anderson et al., 1997; Araibi et al., 2004).

Despite its small size, BPV E5 has wide pleiotropic effects. It induces growth in low serum and in suspension, prevents contact inhibition, down-regulates gap junction communication (GJIC) (for reviews, see DiMaio and Mattoon, 2001, and Venuti and Campo, 2002, and references therein), induces swelling and fragmentation of the GA and vacuolisation of the cytoplasm

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(Ashrafi et al., 2002) and inhibits the expression of MHC class I on the cell surface (Ashrafi et al., 2002; Marchetti et al., 2002). BPV-1 E5 interacts with, and activates, the PDGF receptor and several other kinases, including c-src; both BPV-1 and BPV-4 E5 bind 16 k ductin/subunit c, a component of the gap junction and of the V0 sector of the vacuolar H⁺-ATPase, and prevent the acidification of the GA. Both the down-regulation of GJIC and the alkalisation of the endomembrane compartments are ascribed to the interaction with 16 k ductin/subunit c and a malfunction of the V-ATPase, but the latter is not always observed (Venuti and Campo, 2002).

In addition to the previously described functions, here we now show that BPV-1 and BPV-4 E5 disturb the actin cytoskeleton and focal adhesions in transformed bovine cells, and increase the motility of transformed mouse cells. However, only BPV-1 E5 confers invasiveness to transformed mouse cells.

2. Materials and methods

2.1. PalF cell lines

PalF are parental primary bovine foetal palate cells, PalF-control cells are partially transformed by BPV-4 E7, HPV-16 E6 and activated ras; PalF-1E5 and PalF-4E5 express either the E5 protein of BPV-1 (1E5) or BPV-4 (4E5), respectively, in addition to the other oncogenes. They have been described before (Ashrafi et al., 2000; Ashrafi et al., 2002). Please note that 4E5 was previously called 4E8. When the two BPV E5 proteins present the same characteristics they will be referred to as E5, otherwise their provenance will be specified.

2.2. NIH 3T3 lines

3T3-pZip cells are NIH 3T3 cells harbouring empty pZip-neo plasmid vector. 3T3-1E5 and 3T3-4E5 cells are NIH 3T3 cells transformed by BPV-1 E5 and BPV-4 E5, respectively, and have been described before (Ashrafi et al., 2002; O'Brien and Campo, 1998). All cell lines were grown in Dulbecco modified Eagles medium (DMEM) (Life Technologies, UK), 10% fetal calf serum (FCS) at 37 °C in 5% CO₂.

2.3. Cytoimmunofluorescence

Cells were grown on glass coverslips (10⁴ cells/coverslip), washed twice with PBS, fixed by 10-min incubation at room temperature with 3% paraformaldehyde and washed three times with PBS. They were permeabilised by 20-min incubation at room temperature with 0.5% Triton X-100 (Sigma, UK) in PBS-1% FCS and washed three times with PBS-1% FCS. Cells were stained with FITC- or TRITC-phalloidin (Sigma) to visualise actin fibres, or reacted with monoclonal antibody V9131 (1/400; Sigma) or monoclonal antibody PXC-10 (1/400; Sigma) to visualise vinculin or paxillin, respectively, and FITC-conjugated anti-mouse secondary antibody (1/200; Sigma). After washes with 1% FCS in PBS, coverslips were mounted in AF1. Fluorescence was analysed using

a Leica DMLB microscope or a Leica TCS SP2 confocal microscope.

2.4. Immunoblotting

Cells were removed from the flasks by trypsinization, washed with PBS, and then lysed in lysis buffer (100 mM Tris-HCl, pH 7.5, 2% SDS, 20% glycerol) containing protease inhibitors (Roche Diagnostic Ltd.). Insoluble material was removed by centrifugation at 20,000 × g. Ten micrograms of lysate were electrophoresed in 4–12% NuPAGE gels (Invitrogen), and proteins transferred to nitrocellulose membrane (Invitrogen) using a semidry blotting apparatus at 20 V/150 A for 1 h. The membranes were blocked for 1 h in 5% milk/TBS/Tween 20 (0.05%) at room temperature before a 2 h incubation with anti-actin AB-1 (1/10,000; Merck Bioscience, UK), anti-vinculin V9131 (1/2000) and anti-paxillin PXC-10 (1/2000) antibodies (from Sigma). An antibody to GAPDH (1/2000; Abcam Ltd, UK) was used to control for the amount of protein. For detection of c-src protein, membranes were probed with anti-total c-src polyclonal antibody SC-18 (1:200; Santa Cruz Biotechnology Inc.), or anti-phosphorylated (active) c-src polyclonal antibody c-src(p416) (1:1000; kind gift of M. Frame). After repeated washing with TBS/Tween 20 (0.05%) the membranes were incubated with either anti-mouse (for actin, vinculin, paxillin and GAPDH) or anti-rabbit (for c-src) Ig-HRP (1/2000; Amersham Pharmacia Biotech, UK) in 5% milk/TBS/Tween 20 (0.05%) for 1 h at room temperature. The membranes were washed three times with TBS/Tween 20 (0.05%) and bound antibody was detected by enhanced chemiluminescence staining (ECL) (Amersham Pharmacia Biotech).

2.5. Motility assay

Cells were grown in DMEM, 10%FCS until confluent. A scratch was made in the cell monolayer with a microlance 3 needle and the cell were photographed in a phase-contrast microscope at 0, 2, 4, 6 and 8 h after scratching. The scratch width in the cell monolayer at 0 time was arbitrarily assigned a value of 1 and the width of each scratch at various time points was measured and expressed as a fraction of 1. At least five measurements per cell line were taken.

2.6. Invasion assays

The ability of cells to invade was measured indirectly by anchorage independence assays and by matrigel invasion. The former assays were carried out as described by Pennie et al., 1993. Briefly, cells (10⁵) were added to 15 ml of 1% Methocel in DMEM, 30% FCS, plated in duplicate in bacterial Petri dishes and left at 37 °C for 12 days before being scored and photographed. At least three assays were performed for cell line.

Inverse invasion assays were carried out as previously described (Hennigan et al., 1994) with exceptions that reduced growth factor matrigel (BD Biosciences) was used. Single cells were seeded at 2 × 10⁴ per Transwell and DMEM above the matrigel contained 10% FCS. Cells were stained with calcein

AM (Scott et al., 2004). Confocal microscopy and quantitation was largely as previously described (Hennigan et al., 1994), with exceptions that a positive pixel is defined as one with an intensity value greater than operator-defined background and that optical sections were scanned at 3 μm intervals. Only cells in the 9 μm section or above were considered invasive for quantitation purposes. A compound image of these sections was quantified (Hennigan et al., 1994) then normalised to the value obtained from the corresponding 0 μm section as a “loading” control. Overall results were determined from three separate assays performed with duplicate samples in each, with the exception of 3T3-4E5 cells, which were only included in two assays. Up to 6 separate serial sections were quantified for each sample.

3. Results

3.1. E5 disturbs and collapses the actin cytoskeleton in PalF cells

PalF-1E5 and PalF-4E5 cells have a very disturbed morphology: they are highly vacuolated and have a swollen, misshapen and often fragmented GA (see Fig. 4 of Faccini et al., 1996, Fig. 1 of Ashrafi et al., 2000 and Fig. 1 of Ashrafi et al., 2002).

To see if E5 expression affected other cell structures, we stained the PalF cell lines for actin fibres with FITC- or TRITC-phalloidin which stains the cytoskeleton. Analysis of the cells revealed that cells expressing E5 had long pseudopods, lamellipods and pronounced ruffles and a profoundly disturbed actin cytoskeleton, with shortened actin fibres that did not appear to reach the cell membrane (Fig. 1A). The shortening of the actin fibres was not observed in PalF cells expressing 4N17S and 4EST, non-transforming mutants of BPV-4 E5, whereas it was present in cells expressing 4N17A, a hyper-transforming mutant of the protein (Fig. 1A). These differences in cell morphology were not due to differences in expression of the various E5 proteins as both wild type E5 and its mutants were expressed at comparable levels (O'Brien et al., 1999). This confirmed that the abnormal morphology of the actin cytoskeleton in E5-expressing cells was due to the transforming action of E5. Furthermore, no obvious abnormalities were observed in the parental non transformed cells or in the transformed PalF-control cells (Fig. 1A; Table 1).

3.2. E5 disturbs focal adhesions in PalF cells

The fibres of the actin cytoskeleton connect with focal adhesions (FA), structures that allow interaction between cells and substrate (Geiger et al., 2001). Vinculin and paxillin are both component of FA (Geiger et al., 2001). To determine whether FA were also affected by E5, cells were stained with antibodies against vinculin or paxillin. In the parental PalF and PalF-control cells, vinculin was clearly located in FA at the periphery of the cell (Fig. 1B). In the PalF-1E5 and PalF-4E5 cells vinculin was detected in internal structures, while small chain-like structures similar to focal complexes (Geiger et al., 2001) were sometimes observed at the cell periphery (Fig. 1B and inset; Table 1). Growing PalF-E5 cells on fibronectin, which, being a ligand for integrins, induces formation of FA, did not lead to the appearance of FA on the cell surface (data not shown). FA, therefore, appeared disturbed in PalF-E5 cells. When PalF cells were co-stained for actin and vinculin, vinculin was found at the end of the shortened actin fibres in E5 cells (Fig. 1C), indicating that the interaction between vinculin and actin fibres was maintained but not on the cell surface. In PalF cells expressing non transforming mutants of BPV-4 E5, the FA resembled those of parental and control cells, whereas in PalF cells expressing the hyper-transforming mutant they resembled those of PalF-E5 cells (Fig. 1C). Similar results were obtained with paxillin, another component of FA (data not shown).

3.3. E5 does not induce degradation of actin or FA components

To see if the disruption of the actin fibres and the disassembly of the FA were due to E5-induced degradation of actin, vinculin or paxillin, as observed for the heavy chain of the major histocompatibility complex (MHC) class I (Ashrafi et al., 2002; Marchetti et al., 2002), cell lysates from the PalF cell lines were analysed for these proteins. No noticeable differences were seen between control cells and E5-expressing cells (Fig. 2). Moreover, the pattern of actin fibres and of FA did not change when E5-expressing cells were incubated with the proteasome inhibitor MG-132 or the calpain inhibitors I and II (calpain degrades FA proteins) (data not shown), confirming that the observed interference with these structures is not due to protein degradation.

Table 1
Characteristics of bovine and murine cells expressing BPV E5

	PalF control cells	PalF-1E5	PalF-4E5	3T3 control cells	3T3-1E5	3T3-4E5
Disturbed cytoskeleton	No	Yes	Yes	No	Yes	Yes
Mis-located vinculin/paxillin	No	Yes	Yes	No	No	No
Anchorage independence	No	Yes	Yes	No	Yes ^a	Yes ^a
Motility	Nd ^b	nd ^b	nd ^b	No	Yes	Yes
Invasion	No ^c	nd ^c	nd ^c	No	Yes	No
pY-c-src	No	Yes	Yes	No	Yes	No

^a Data from O'Brien and Campo, 1998.

^b Could not be determined because of monolayer lifting.

^c Could not be determined because of cell death in matrigel.

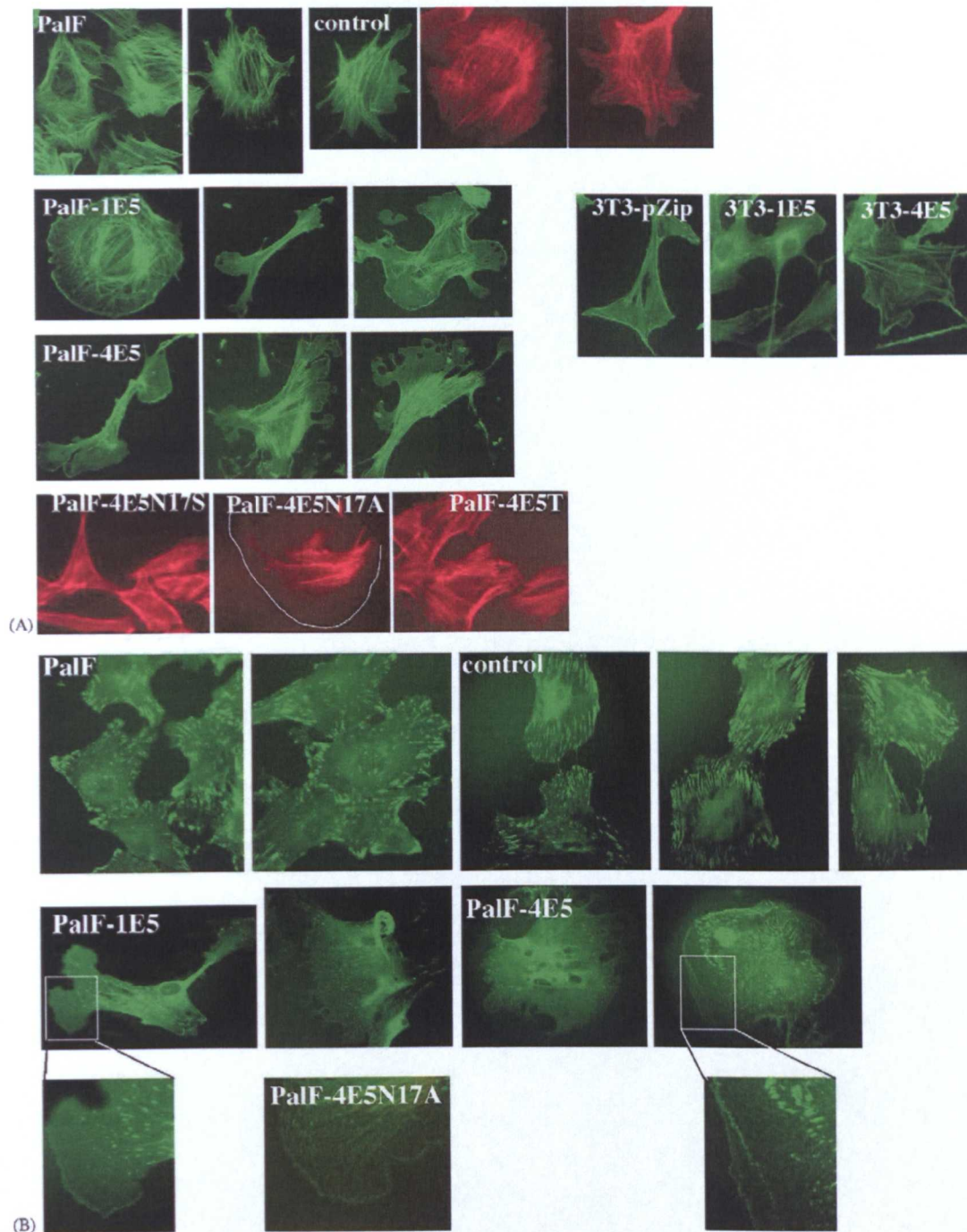


Fig. 1. Morphological changes in cells expressing BPV-1 or BPV-4 E5 proteins. (A) PalF-E5 cells and PalF-4E5N17A cells have shortened actin fibres and large lamellipodia protrusions. Parental PalF cells, control cells and cells expressing non-transforming mutants of BPV-4 E5 have a well organised cytoskeleton. 3T3-E5 cells have a collapsed actin cytoskeleton, while control 3T3Zip cells have a well organised cytoskeleton. Cells were stained with FITC-phalloidin (green) or TRITC-phalloidin (red) and analysed using a Leica DMLB microscope. TRITC-phalloidin was used to permit double fluorescence analysis (see panel C). The white line in the 4E5N17A panel delineates the outer margin of the cell. The large size of the PalF-1E5, PalF-4E5 and PalF-4E5N17A cells precludes the imaging of a larger field without loss of resolution, but all PalF E5 cells present a morphology similar to the one shown here for single cells. Magnification 400 \times . (B) PalF-E5 and PalF-4E5N17A cells do not have focal adhesions on their surface. Cells were stained with mAb V9131 (1/400) to visualise vinculin, and FITC-conjugated anti-mouse secondary antibody (1/200). Cells were analysed using a Leica DMLB microscope. The boxed areas in the 1E5 and 4E5 panels are enlarged in the panels below to show the absence of focal adhesions on the cell surface and the presence of focal complex-like structures. An equivalent area is enlarged for PalF-4E5N17A cells. Magnification 400 \times . (C) PalF-E5 cells have vinculin at the tip of the short actin fibres. Cells were stained for vinculin as in (B), and with TRITC-phalloidin to visualise actin fibres as in (A). Cells were analysed in a Leica TCS SP2 confocal microscope and images merged using the accompanying software. Magnification 400 \times . The images of 1E5 and 4E5 cells are at higher magnification (800 \times) to show the interaction between vinculin and actin at the tip of the shortened actin fibres. Likewise, the images of 4E5N17S and 4E5T are at higher magnification (800 \times) to show the presence of vinculin at the cell periphery. The white lines in the 1E5 and 4E5 panels delineate the outer margin of the cells. For cell nomenclature, see Section 2.

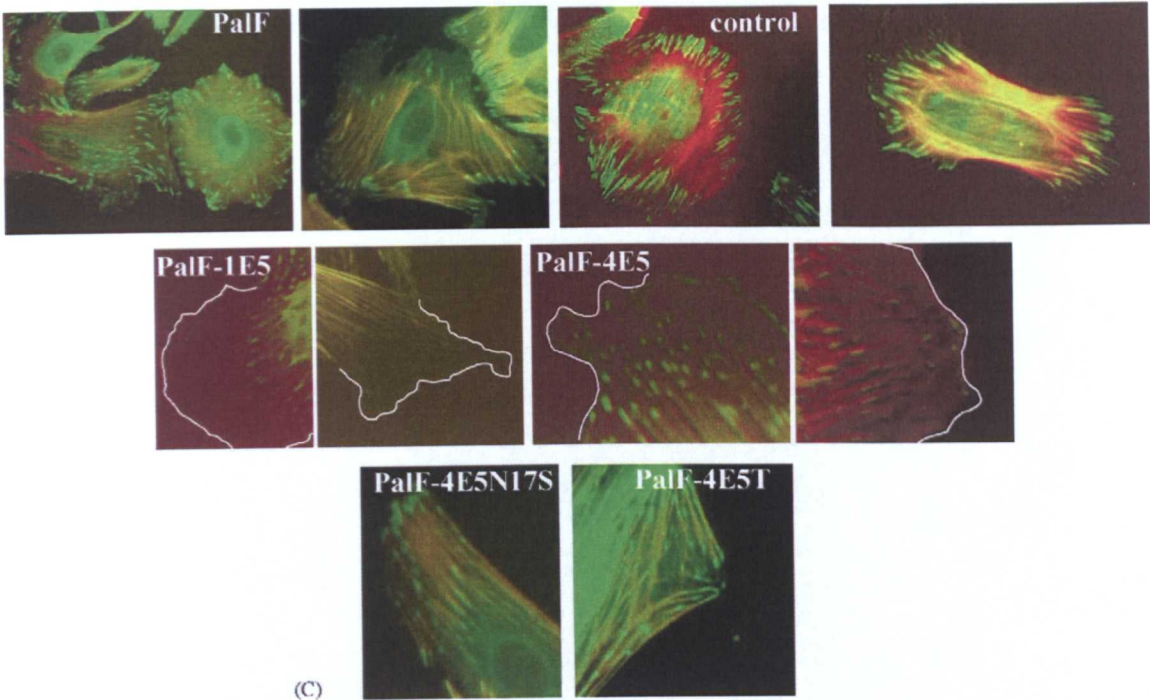


Fig. 1. (Continued).

3.4. E5 enables PalF cells to grow independently of substrate

Adhesion to substrate is controlled by FA and disruption of FA promotes cell growth independent of substrate (Carragher and Frame, 2004). We have already reported that both PalF and NIH 3T3 cells expressing BPV E5 are capable of anchorage-independent growth (O'Brien et al., 1999; O'Brien and Campo, 1998). We confirm that this is indeed the case: PalF-1E5, PalF-4E5 and PalF-4N17A cells grew in methocel, whereas neither

parental PalF, control or PalF-4E5T cells were capable of doing so (Fig. 3; Table 1). The disruption of FA brought about by E5 is likely to contribute to the ability of the cells to grow in an anchorage independent fashion.

3.5. NIH 3T3 cells expressing BPV E5 are more motile

The cytoskeleton and the FA regulate cell shape and cell motility (Geiger et al., 2001). Given the profusion of pseudopods, lamellipods and membrane ruffles and the absence of FA in PalF-1E5 and PalF-4E5 cells, we sought to analyse their motility in the scratch wound assay. However, the assay could not be performed with the PalF cell lines because the cell monolayers would lift and float instead of scratching (Table 1). Therefore, we decided to analyse NIH 3T3 cells transformed by BPV E5 (O'Brien and Campo, 1998; O'Brien et al., 2001). First we analysed the status of the actin cytoskeleton of these cells, as we did for the PalF cell lines. Following staining with FITC-phalloidin, the actin cytoskeleton was normally organised in 3T3-pZip cells, but was collapsed in the 3T3-E5 cells, with actin located cortically and long pseudopodes (Fig. 1A; Table 1). The morphology of the 3T3-E5 cells was however less disturbed that that of PalF-E5 cells. Despite the collapse of the actin cytoskeleton, as in the PalF cells, there were no differences in the levels of actin between control and E5 cells (data not shown), and no disruption of FA was observed in the 3T3-E5 cells with either the paxillin or the vinculin antibody (data not shown; Table 1). In the scratch assay, the 3T3-E5 cells repopulated and closed the wound faster than control cells (Fig. 4A and B; Table 1), indicating that BPV E5 can increase cell motility, at least in NIH 3T3 cells. Increase motility however appears to be independent of the status of FA in NIH 3T3 cells.

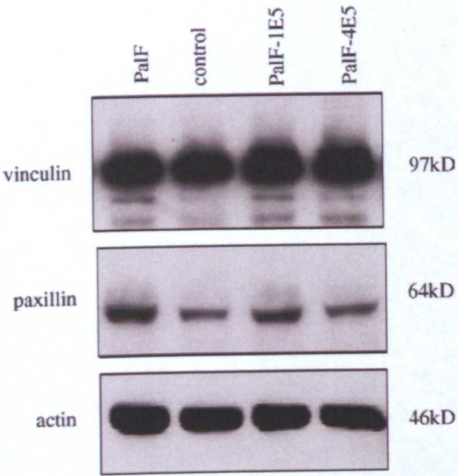


Fig. 2. Actin and focal adhesion components in PalF-E5 cells. E5 does not induce the degradation of actin, vinculin or paxillin. Membranes with proteins from PalF cells were probed with anti-actin mAb AB-1 (1/20,000), anti-vinculin mAb V9131 (1/2000) and anti-paxillin mAb PXC-10 (1/2000) and then anti-mouse Ig-HRP (1/2000). Bound antibody was detected by enhanced chemiluminescence staining.

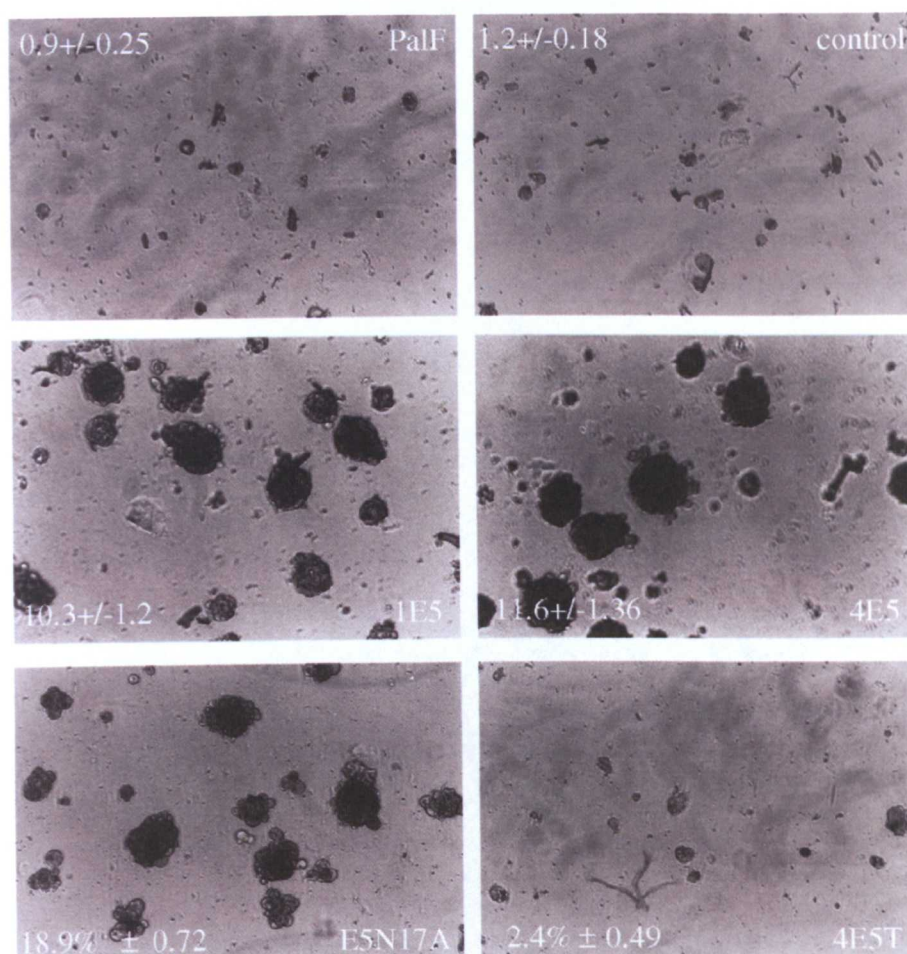


Fig. 3. PalF-E5 cells and PalF-4E5N17A cells, but not PalF-4E5T or control cells, grow in Methocel. Cells were grown in 1% Methocel in DMEM, 30% FCS and photographed after 12 days of growth ($\times 40$). At least three assays were performed for cell line. Numbers in the panels indicate the average % (\pm S.D.) of total cells seeded into each plate on day 0 (10^5).

3.6. NIH 3T3-1E5 cells are more invasive

Next we wished to analyse whether, in addition to increased motility, E5 transformed cells would also be more invasive. To this end we employed the three-dimensional matrigel invasion assay (Hennigan et al., 1994; Scott et al., 2004). Both control and PalF-E5 cells died in the matrigel and, therefore, their invasion ability could not be assessed (Table 1). Thus, as for the motility assay, we performed the test on the NIH 3T3 cells. Parental 3T3-pZip cells did not invade the matrigel, whereas 3T3-1E5 cells were capable of invasion and were detected at least 9 μ m from the filter (Fig. 5). Surprisingly, however, 3T3-4E5 cells were not capable of invading and were not detected beyond the upper surface of the filter (Fig. 5; Table 1). The inability of 3T3-4E5 cells to invade the matrigel was not due to poor E5 expression, as E5 was expressed at detectable levels (O'Brien et al., 2001), or to death as the cells were detected on the filter (Fig. 5).

3.7. c-src is hyperphosphorylated in E5-expressing cells

The cellular protein c-src is a tyrosine kinase that modulates the actin cytoskeleton and cell adhesions (Frame et al.,

2002). Activation of the catalytic activity of c-src through phosphorylation initiates a signal transduction cascade that induces adhesion turnover, actin re-modelling, cell migration and invasion (Frame, 2002, 2004; Frame and Brunton, 2002). Given the ability of PalF E5 cells to grow in suspension and the disrupted state of their cytoskeleton and FA, and the greater motility of 3T3-1E5 and 3T3-4E5 cells and invasion properties of the 3T3-1E5 cells, we examined the phosphorylation status of c-src in these cells. We found that c-src was more highly phosphorylated in transformed PalF-1E5 and PalF-4E5 cells and in 3T3-1E5 cells than in the parental or control cells (Fig. 6A and B; Table 1). On the contrary, c-src was hypophosphorylated in 3T3-4E5 cells which were incapable of invasion. Thus, a precise correlation between c-src activation and cell morphology and behaviour could not be established.

Cytoskeletal rearrangements and cell motility can be controlled by the small GTPase family members (Nobes and Hall, 1999). We analysed levels of RhoA, Rac and Cdc42 in both PalF and 3T3 E5-expressing cells but did not find any difference with control cells (data not shown). We were unable to detect activated Rho GTPases in our cellular samples by using spe-

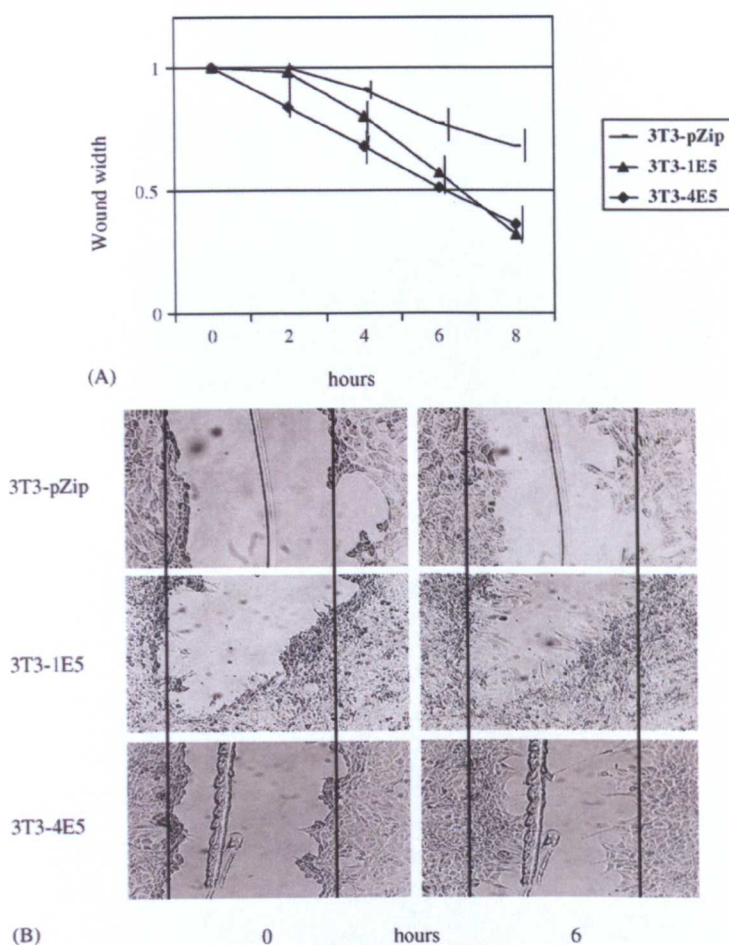


Fig. 4. 3T3-E5 cells are more motile. (A) Cell monolayers were scratched (wounded) and the scratch width at 0 time was arbitrarily assigned a value of 1 and the width of each scratch at various time points was measured and expressed as a fraction of 1. At least five measurements per cell line were taken. (B) examples of wound healing of 3T3-pZip, 3T3-1E5 and 3T3-4E5 cells. The black vertical lines indicate the maximum width of the wound. Images were taken at 0 and 6 h.

cific pull-down assays, and therefore, the activation status of the small GTPases remains to be elucidated.

Table 1 shows a summary of the characteristics of PalF and 3T3 cells.

4. Discussion

We describe here further characteristics of morphological cell transformation brought about by the E5 oncoproteins from one cutaneous (BPV-1) and one mucosal BPV (BPV-4) in two different cell systems, primary bovine PalF cells (the natural host of BPV) and established murine NIH 3T3 cells. While PalF cells need several viral (E5, E6 and E7) and cellular (activated ras) oncoproteins to achieve transformation (Pennie et al., 1993; O'Brien et al., 1999), mouse NIH 3T3 cells can be easily transformed by a variety of agents, including BPV E5 (Schiller et al., 1986; O'Brien and Campo, 1998). Despite these differences, the use of the two different cell systems has provided information that could not be obtained in one system alone.

4.1. E5 and PalF cell morphology

Expression of either BPV-4 or BPV-1 E5 brings about profound modifications in the actin cytoskeleton, with "shortened"

actin fibres, without any noticeable reduction in the overall amount of actin. These cytoskeletal rearrangements are accompanied by increased formation of lamellipodia, membrane ruffles and filopodia.

Both E5 proteins induce also the relocation, but not degradation, of vinculin and paxillin (both components of FA) at the end of the shortened actin fibres. This destabilisation of FA, in addition to E5-induced up-regulation of cyclin A (O'Brien et al., 1999), is likely to underlie the ability of E5-transformed cells to grow independently of anchorage to the substrate (O'Brien et al., 1999; Ashrafi et al., 2000).

The cytoskeleton and FA are not the only cell structures affected by E5 in PalF cells. The Golgi apparatus (GA) is enlarged and often fragmented, and the cytoplasm is highly vacuolated (Faccini et al., 1996; Ashrafi et al., 2000, 2002). We have attributed these modifications of the endomembrane compartments to their alkalisation brought about by a malfunction of the H^+ V-ATPase due to the interaction between E5 and 16k subunit c, as similar changes in the GA and cytoplasm are caused in control cells by the ionophore monensin, which inhibits the proton pump (Marchetti et al., 2002). However, treatment of PalF control cells with monensin did not cause changes in FA, and the changes in actin fibres were dissimilar to those observed

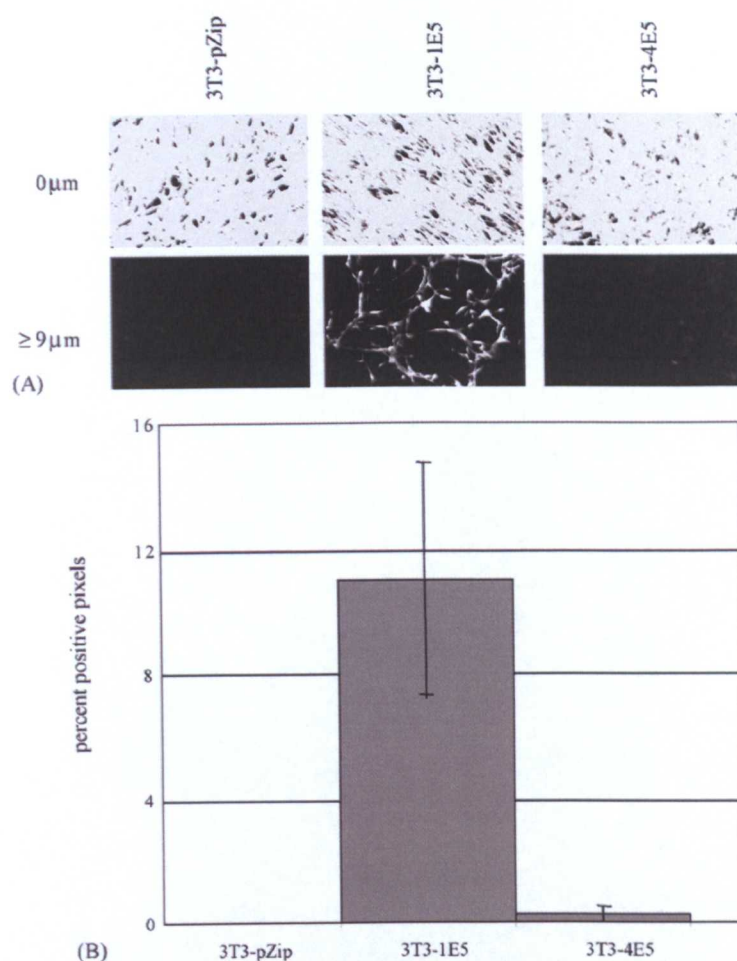


Fig. 5. 3T3-1E5 cells are invasive. (A) Confocal microscope images of calcein-stained cells in the invasion assay. The upper row shows cells on the underside of the filter and the lower row shows the corresponding compound images of transverse sections (Section 4 and above), showing cells that have invaded greater than 9 μm into the matrigel. (B) Quantitation of invading cells. The units for the y-axis are percent positive pixels after normalization for loading (see Section 2 for details).

in E5 cells (data not shown). We, therefore, conclude that the rearrangement of the actin cytoskeleton and the destabilisation of the FA are caused by E5 through a different pathway(s) than the targeting of the proton pump.

PalF cells transformed by the complete BPV-4 genome and activated *ras* also have a distorted shape with enlarged cytoplasm and vacuoles (Jaggar et al., 1990). Although not experimentally proven, it is reasonable to assume that these changes are due, at least in part, to the E5 protein.

4.2. E5, cell motility and invasiveness in NIH 3T3 cells

BPV E5 induces anchorage independence in PalF cells as described earlier (Pennie et al., 1993; O'Brien et al., 1999) and confirmed here. Despite their ability to grow when deprived of substrate, the PalF-E5 cells were not capable of surviving in the matrigel and thus their invasion ability could not be assessed. The reason for the failure of the PalF E5 cells to survive in the matrigel may reside in their inability to stay alive in the absence of contact with surrounding cells. Likewise, we could not establish the effects of E5 on motility in PalF cells because of the lifting of the monolayer when scratched. Given the impossibility

to assess the motility and invasiveness of PalF cells, we decided to investigate these properties in NIH 3T3 cells transformed by either BPV-1 or BPV-4 E5 (O'Brien and Campo, 1998; O'Brien et al., 2001). The analysis of these transformed cells proved fruitful and in addition revealed differences between BPV-1 E5 and BPV-4 E5. While both 3T3-1E5 cells and 3T3-4E5 cells had a disturbed actin cytoskeleton and showed enhanced motility, only 3T3-1E5 cells proved invasive, suggesting a greater transformation potential of BPV-1 E5 than BPV-4 E5 in NIH 3T3 cells.

4.3. Activation of *c-src*

The cellular tyrosine kinase *c-src* modulates the actin cytoskeleton and cell adhesions (Frame, 2002). Phosphorylation of *c-src* activates a signal transduction cascade that induces adhesion turnover, actin re-modelling, cell migration and invasion (Frame, 2002; Frame and Brunton, 2002). BPV-1 E5 activates *c-src* in NIH 3T3 cells (Suprynowicz et al., 2002) and, in agreement with these observations, we found that *c-src* is hyperphosphorylated (activated) in PalF-1E5 and PalF-4E5 cells and in 3T3-1E5 cells. The activation of *c-src* may be due to the E5-induced alkalisation of the GA (Schapiro et al., 2000), as

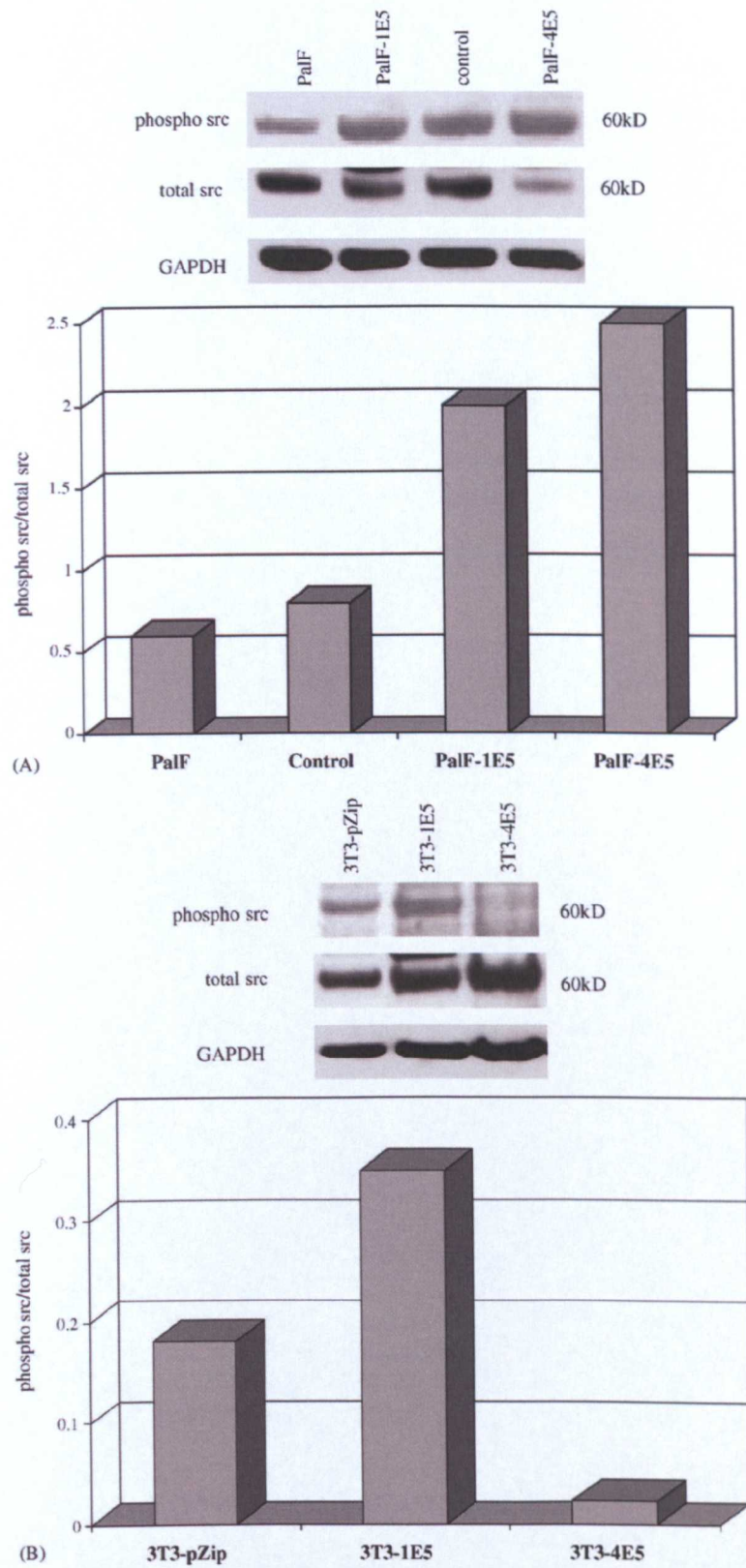


Fig. 6. C-src is hyperphosphorylated in E5-expressing cells. (A) PalF cells. Top panel, membranes with cell proteins were probed with anti-phosphorylated (active) c-src polyclonal antibody c-src(p416) (1:1000) or anti-total c-src polyclonal antibody SC-18 (1:200), and then anti-rabbit Ig-HRP (1/2000). Bound antibody was detected by enhanced chemiluminescence staining. Bottom panel, the western blots were scanned in a MagicScan and quantified using the Image Quant 5.3 Software (Molecular Dynamics). The ratio of phosphorylated c-src over total c-src is shown. The experiment was repeated two times with essentially identical results. (B) NIH 3T3 cells. Top panel, membranes with cell proteins were probed as in (A) top panel, and the western blots were scanned and quantified as in (A) bottom panel.

speculated by Suprynowicz et al. (2002) and may contribute to the transformed status of the cells observed here. However, c-src is not hyperphosphorylated in 3T3-4E5 cells, highlighting another difference between BPV-1 E5 and BPV-4 E5. As there is not a clear-cut correlation between c-src activation and cell morphology and behaviour, the precise consequence of c-src activation cannot be unequivocally established. The reason why BPV-4 E5 induces phosphorylation of c-src in PalF cells but not in NIH 3T3 cells is not known. It is to be noted however that BPV-4 E5 is not alone in exhibiting cell-specific effects: the oncoprotein v-fos easily transforms rodent fibroblasts but not human cells, although it makes the latter invasive (Scott et al., 2004, and references therein).

4.4. Cell morphology and viral proteins

It is interesting that the Nef protein of HIV, with which E5 shares the ability to bind components of the H⁺ V-ATPase and to down-regulate surface MHC class I (Kasper and Collins, 2003; Lu et al., 1998), also induces rearrangements of the actin cytoskeleton and the formation of lamellipodia and filopodia (Fackler et al., 1999). Expression of SV40 small t in epithelial cells brings about similar morphological and structural changes (Nunbhakdi-Craig et al., 2003), including the formation of numerous giant cells, as in our PalF system (Ashrafi et al., 2000; Faccini et al., 1996). The changes in cell shape and cytoskeleton observed in these three different viral protein systems resemble those associated with activation of small GTPases, particularly Rac (Nobes and Hall, 1999). Indeed this is the case for Nef (Fackler et al., 1999), and both Rac and Cdc42 are over-expressed in small t-expressing cells (Nunbhakdi-Craig et al., 2003). Like Nunbhakdi-Craig et al. (2003), we could not detect activation of the small GTPases, but, unlike those authors, did not find any change in their level of expression (data not shown) and are, therefore, unable to conclude that E5 activates them.

4.5. Conclusions

As shown here and as reported before, BPV E5 has a global effect on the morphology and behaviour of PalF cells: cells are vacuolated with a distended and fractured Golgi apparatus, have a rearranged actin cytoskeleton and disassembled focal adhesions, grow in low serum and independently of substrate, and protein traffic to the cell periphery is disturbed. Furthermore E5 affects several signal transduction pathways and cell cycle regulators. The small size of E5 suggests that these multiple effects are due to a critical interaction(s) between E5 and a crucial cellular partner(s).

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Appendix (f)

ORIGINAL ARTICLE

The E5 protein of BPV-4 interacts with the heavy chain of MHC class I and irreversibly retains the MHC complex in the Golgi apparatus

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BPV-4 E5 inhibits transcription of the bovine MHC class I heavy chain (HC) gene, increases degradation of HC and downregulates surface expression of MHC class I by retaining the complex in the Golgi apparatus (GA). Here we report that transcription inhibition can be alleviated by interferon treatment and the degradation of HC can be reversed by treatment with inhibitors of proteasomes and lysosomes. However, the inhibition of transport of MHC class I to the cell surface is irreversible. We show that E5 is capable of physically interacting with HC. Together with the inhibition of the vacuolar ATPase (due to the interaction between E5 and 16k subunit c), the interaction between E5 and HC is likely to be responsible for retention of MHC class I in the GA. C-terminus deletion mutants of E5 are incapable of either downregulating surface MHC class I or interacting with HC, establishing that the C-terminus domain of E5 is important in the inhibition of MHC class I.

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2005). Similarly, the E5 oncoprotein of both BPV-1 and BPV-4 inhibits the transport of MHC class I to the cell surface and retains the complex in the Golgi apparatus (GA) (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002). BPV-4 E5-induced downregulation of MHC class I takes place not only in cultured cells but crucially also in naturally occurring papillomas (Araibi *et al.*, 2004). We have argued that the downregulation of MHC class I is an important step in the establishment and persistence of papillomavirus infections and neoplastic progression of premalignant lesions to squamous carcinoma (Ashrafi *et al.*, 2005). While both BPV and HPV E5 proteins retain MHC class I in the GA, only BPV E5 induces a general downregulation of MHC class I expression (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002). Here we show that BPV-4 E5-induced transcriptional inhibition of the MHC class I heavy chain (HC) gene and degradation of the HC peptide can be alleviated by treatment with interferon (IFN) or proteasome and lysosome inhibitors respectively, but that MHC class I transport to the cell surface is irreversibly compromised, probably due to a physical interaction between E5 and HC.

Introduction

MHC class I presents antigenic peptides, including viral peptides, to cytotoxic T lymphocytes (CTL), effector cells capable of recognizing and destroying transformed or infected cells. MHC class I is therefore a central player of the adaptive immune response. Numerous viruses have developed the ability of downregulating MHC class I (Piguet, 2005) either through direct interaction between viral proteins and MHC class I, as in the case of HIV and HCMV (Chevalier *et al.*, 2002; Furman *et al.*, 2002; Tirabassi and Ploegh, 2002; Williams *et al.*, 2002; Kasper and Collins, 2003), or indirectly, as in the case of Adenovirus (Ad) 12 (Piguet,

Results

The downregulation of MHC class I by BPV E5 takes place at several levels: transcription of the HC, degradation of the HC and transport of the MHC complex to the cell surface (Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002).

Transcription of the HC of the MHC class I complex in E5-expressing cells increases with IFN treatment

In PalF-4E5 cells, including cells expressing N17A, a hypertransforming mutant of BPV-4 E5 (4E5), the levels of the HC RNA are drastically reduced compared to control cells (Figure 1a, white bars). To investigate whether the reduction in HC RNA was due to transcriptional repression or to increased degradation of the RNA, pBoLa-Luc, containing the reporter gene for luciferase under the transcriptional control of the promoter/enhancer of a bovine MHC class I HC gene, was transiently introduced into PalF cells along with pZip-4E5, a plasmid expressing BPV-4 E5. Expression

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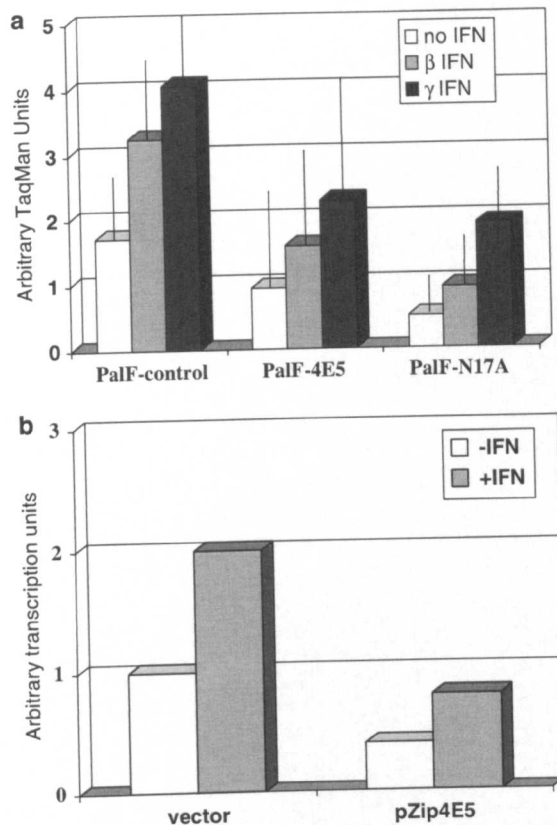


Figure 1 BPV-4 E5 inhibits transcription from the BoLa promoter. Quantitative RT-PCR for MHC class I HC RNA was performed on RNA from PalF control cells and PalF-4E5 cells (a), before (white bars) or after treatment with 500 U/ml β IFN (grey bars) or 250 U/ml γ IFN (black bars). The extent of amplification was normalized to that of the actin RNA and is plotted as arbitrary units. The panel shows the averages with standard deviations of at least three experiments. Both β IFN and γ IFN increased transcription of HC RNA in control and E5 cells. (b) BoLa promoter transcriptional activity in PalF parental cells transiently cotransfected with pBoLa-Luc and pZipneo empty vector, or with pBoLa-Luc and pZip-4E5, either without (white bars) or with 250 U/ml γ IFN treatment (grey bars). Luciferase activity is plotted as arbitrary transcription units, with the activity of pBoLa in PalF parental cells taken as 1. The values are the average of three almost identical measurements. γ IFN increases the transcriptional activity of pBoLa also in the presence of E5.

of luciferase was less than half the level than when pBoLa-Luc was cotransfected with an empty pZip vector (Figure 1b, white bars). This result indicates that the reduction in HC RNA in PalF-4E5 cells is due to the E5-induced inhibition of the transcriptional promoter of the HC gene, similar to the repression of class I transcription by Ad12 E1A (Piguet, 2005).

The transcription of genes encoding MHC HCs is stimulated by both class 1 and class 2 IFNs (Agrawal and Kishore, 2000). The bovine HC promoter used in these experiments contains responsive elements for both β IFN and γ IFN (data not shown). Treatment of the PalF control and PalF-4E5 cell lines with either β IFN or γ IFN lead to a noticeable increase of HC RNA: between

2- and 3-fold in control cells, and between 5 and 10 fold in the PalF-4E5 cells (Figure 1a, grey and black bars). Similar increases in transcription of the HC gene upon IFN treatment were observed in parental PalF cells (data not shown). However, despite the IFN-induced increase in HC gene transcription, the levels of HC RNA in the PalF-4E5 cells were 2- to 3-fold lower than in the control cells. Additionally, γ IFN-treatment stimulated the expression of luciferase from pBoLa-Luc by approximately 2-fold both in the presence or absence of 4E5 (Figure 1b, grey bars). Even with γ IFN-treatment, in cells expressing 4E5 luciferase expression did not achieve the levels observed in cells that did not express E5 (Figure 1b). Treatment with IFN did not increase transcription of the 4E5 gene (data not shown).

We conclude from these results that 4E5 inhibits transcription of cattle class I HC genes, and that IFN treatment rescues transcription, but without completely overcoming 4E5-induced repression.

Lysosome and proteasome inhibitors increase the stability of the HC in E5-expressing cells

MHC class I stability is regulated by proteasomes and lysosomes, which degrade mis-located or mis-folded MHC class I molecules (Hughes *et al.*, 1997; Hewitt *et al.*, 2002; Bartee *et al.*, 2004). The levels of HC protein are extremely low in PalF-4E5 cells, but increase when transcription of the HC gene is stimulated by IFN treatment, without however reaching the levels of HC in IFN-treated control cells (Figure 2). To investigate further the cause of this reduction in HC protein, the control and 4E5 cell lines were treated with inhibitors of lysosomes (bafilomycin or ammonium chloride) or of proteasomes (MG132 or ALLN). Levels of MHC class I were assessed by immunoblotting. Treatment increased the amounts of HC in control PalF cells but only marginally in PalF-4E5 cells (Figure 2, lanes with no IFN), probably because of the preceding block of HC gene transcription. Given that the transcriptional block could be relieved by IFN, we treated cells with both IFN and inhibitors. After the combined treatment, the level of HC protein in PalF-4E5 cells was comparable with that of control PalF cells (Figure 2). We conclude therefore that in 4E5 cells, as in control cells, the degradation of the HC protein via the lysosomes and proteasomes can be abrogated by treatment with inhibitors.

Transport of MHC class I complex to the cell surface is irreversibly inhibited by E5

To see whether the increase in MHC class I HC induced by treatment with IFN and lysosome or proteasome inhibitors resulted in increased levels of MHC class I complex on the cell surface, we performed flow cytometry analyses on the treated cells. Treatment with IFN + bafilomycin or IFN + MG132 increased the amount of surface MHC class I in control cells by 5- to 6-fold, but not in PalF-4E5 cells, where the increase was so negligible that the shift in forward fluorescence

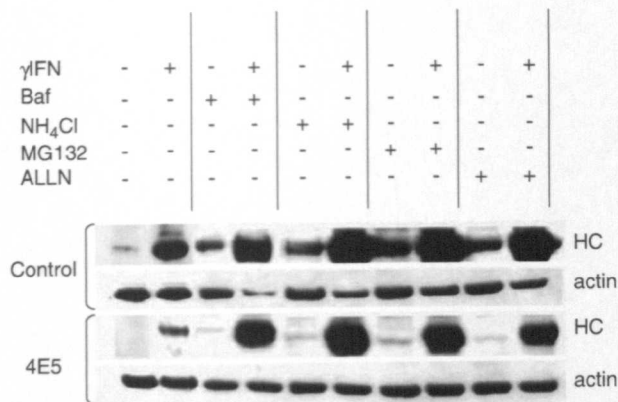


Figure 2 Proteasome and lysosome inhibitors prevent degradation of MHC class I HC in PalF control and PalF-4E5 cells. Cells were left untreated or treated with either 250 U/ml γ IFN for 48 h, the lysosome inhibitors Bafilomycin A1 (1 μ M) or NH₄Cl (40 mM) for 24 h, or the proteasome inhibitors MG-132 (5 μ M) or ALLN (100 μ M) for 8 h. For combined treatment with γ IFN and inhibitors, lysosome inhibitors were added for the last 24 h of γ IFN treatment, and proteasome inhibitors for the last 8 h. Protein lysates were probed with mAb IL-A88 against HC, or with mAb AB-1 against actin. Combined γ IFN and inhibitors treatment increases levels of MHC class I HC in both control and E5 cells. The experiment shown is representative of at least three experiments giving essentially the same results.

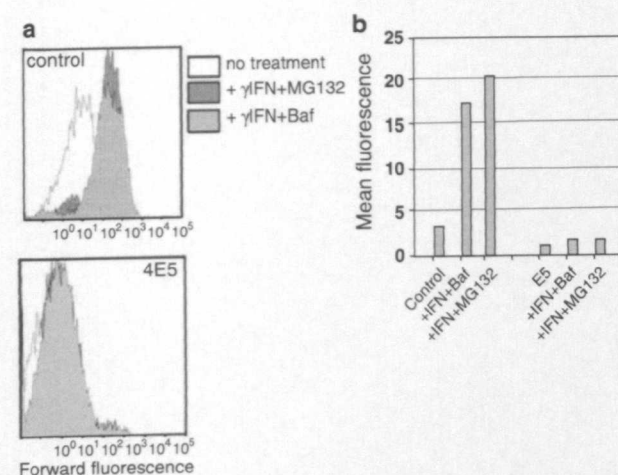


Figure 3 MHC class I transport to the cell surface is irreversibly inhibited by BPV-4 E5. Cells were treated with γ IFN + MG-132 or γ IFN + Bafilomycin A1, as in Figure 2, and expression of surface MHC class I was analysed by flow cytometry with mAb IL-A19. (a) FACS profiles of control and 4E5 cells with no treatment (white), γ IFN + MG-132 (dark grey) and γ IFN + Bafilomycin A1 (light grey). (b) The extent of surface MHC class I expression is plotted as mean forward fluorescence. In control cells, combined γ IFN and inhibitors treatment increases surface MHC class I, whereas in E5 cells, surface MHC class I does not increase. The experiment shown is representative of three experiments giving essentially identical results.

could not even be detected (Figure 3). We conclude that the 4E5 protein prevents the traffic of MHC class I complex to the cell surface in an irreversible manner.

E5 and HC protein interact physically

We have shown that the retention of MHC class I in the GA is due at least in part to the alkalization of the organelle (Marchetti *et al.*, 2002), attributed to the binding of E5 to the 16k subunit c of the vacuolar H⁺ ATPase (Goldstein *et al.*, 1991). However, the almost complete colocalization of 4E5 and the residual MHC class I in the GA (Marchetti *et al.*, 2002) would suggest an interaction between 4E5 and the complex. To investigate if any such interaction exists, we performed coimmunoprecipitation experiments both *in vitro* and *in vivo*. 4E5 and the bovine HD6 HC (Ellis *et al.*, 1996; Gaddum *et al.*, 1996) were separately transcribed/translated *in vitro* in the presence of ³⁵S-labelled methionine (Faccini *et al.*, 1996) and then kept separately or mixed together. The proteins were precipitated either with Ab 274 against the C-terminus of BPV-4 E5 (Anderson *et al.*, 1997; Araibi *et al.*, 2004) or mAb IL-A88 against bovine HC. There was no precipitate in the absence of antibody (Figure 4c, lanes 3, 5, 7); the anti-E5 antibody precipitated E5 but not HD6 HC (Figure 4a, lanes 1, 5), and the mAb IL-A88 precipitated HC but not E5 (Figure 4c lanes 4, 6). However, when the two proteins were mixed together, they coprecipitated with either antibody (Figure 4a, lane 2 and 4e, lane 3), indicating that E5 and HC interact.

To see whether the interaction between E5 and HC was specific for a particular MHC haplotype, we repeated the experiment with a different HC, JSP.1 (Pichowski *et al.*, 1996). Also in this case, E5 and JSP.1, HC coprecipitated when incubated with mAb IL-A88 (Figure 4c, lane 8), showing that interaction is not restricted to certain MHC alleles.

To confirm that E5 and HC interact also in cells, coimmunoprecipitation experiments were performed in PalF control cells and in PalF-4E5 cells. Both cell lines were treated with IFN and MG132 as described above to bring the amount of HC in PalF-4E5 cells to detectable levels. Protein lysates from both cell lines were incubated with Ab 274 and the immunoprecipitates were run on gel and immunoblotted with mAb IL-A88. A band of the appropriate MW for HC (approximately 45 kDa) was visible in a Coomassie blue stained gel in the immunoprecipitate from PalF-4E5 cells, which was not visible in the immunoprecipitated from control cells (Figure 4d). This band reacted with mAb IL-A88 and corresponded to bona fide HC detected by direct immunoblotting of protein lysates (Figure 4d). Despite the combined IFN + bafilomycin treatment, the HC band coprecipitated with E5 is faint because the treatment does not increase expression of E5 (data not shown) and E5 is expressed at almost undetectable levels (O'Brien *et al.*, 1999, 2001); therefore, the amount of E5 is limiting for the amount of bound HC. However, the reactivity with mAb IL-A88 specific for bovine HC only in E5-expressing cells, the correspondence of the band with bona fide HC and the results obtained *in vitro*, establish the identity of this band as MHC class I HC and confirm that the interaction between E5 and HC takes place also in cells.

E5 inhibits MHC class I transport and binds to MHC HC via its C-terminus

In a study of the cell transforming properties of BPV-4 E5 (O'Brien *et al.*, 1999), we established that mutation of the asparagine residue 17 to tyrosine (N17Y) lead to loss of cell transformation and so did deletion of the C-terminus (E5T) of the protein. On the contrary, mutation of N17 of BPV-4 E5 to alanine (N17A) resulted in a hyper-transforming mutant. The ability to downregulate MHC class I expression cosegregated with the ability to transform cells (Ashrafi *et al.*, 2002; O'Brien and Campo, 2003). BPV E5 mutants that did not transform did not downregulated MHC class I as assessed by flow cytometry (Figure 5a) and by immunofluorescence (Figure 5b), and both the total and cell surface levels of MHC class I were similar to those of control cells. In contrast, the hyper-transforming mutant of BPV-4 E5 downregulated surface MHC class I to the same extent as wild-type E5 (Figure 5a). To investigate whether the BPV-4 E5 mutants were capable of interacting with HC we performed coimmunoprecipitation experiments *in vitro* as above. Both N17Y and N17A were coprecipitated with HC by Ab 274 (Figure 4a, lanes 6, 8) and mAb IL-A88 (Figure 4e, lanes 5, 7), while E5T was not coprecipitated with HD6 HC by mAb IL-A88 (Figure 4e, lane 9). E5T lacks the C-terminal domain and therefore could not be precipitated by Ab 274. As E5 is tagged at its N-terminus with the HA epitope, we performed coimmunoprecipitation experiments with mAb HA.11 raised against the HA epitope. mAb HA.11 coprecipitated E5 and HC, although not as effectively as Ab 274, (Figure 4b, lanes 1, 2) but did not coprecipitate HC with E5T (Figure 4b, lanes 3, 4). These data indicate that the interaction between E5 and HC takes place via the C-terminus of E5. To confirm this, we performed competition experiments with a well-characterized peptide corresponding to the C-terminus of E5, the ability of which to compete with E5 had been validated in immunocyto- and immunohisto-staining experiment (Pennie *et al.*, 1993; Anderson *et al.*, 1997; Araibi *et al.*, 2004). The peptide did successfully compete with E5 in coimmunoprecipitation experiments (Figure 4e, lane 11); its addition to the reaction prevented the interaction between E5 and HD6, proving conclusively that the C-terminus of E5 interacts with MHC class I HC.

Three conclusions can be reached from these experiments: one, binding of E5 to HC is *necessary* for downregulation of MHC class I as shown by E5T, but not *sufficient* as shown by N17Y; two, the nature of residue 17 is not important for the interaction with HC; and three, the C-terminus of E5 is responsible for the interaction between E5 and HC.

Discussion

The E5 oncoprotein of BPV-4 has a profound effect on MHC class I expression and transport. E5 disruption of the normal metabolism of MHC class I takes place at

several levels: transcription, protein degradation and complex transport.

Inhibition of transcription of the MHC class I HC gene by BPV-4 E5 can be alleviated by IFN treatment

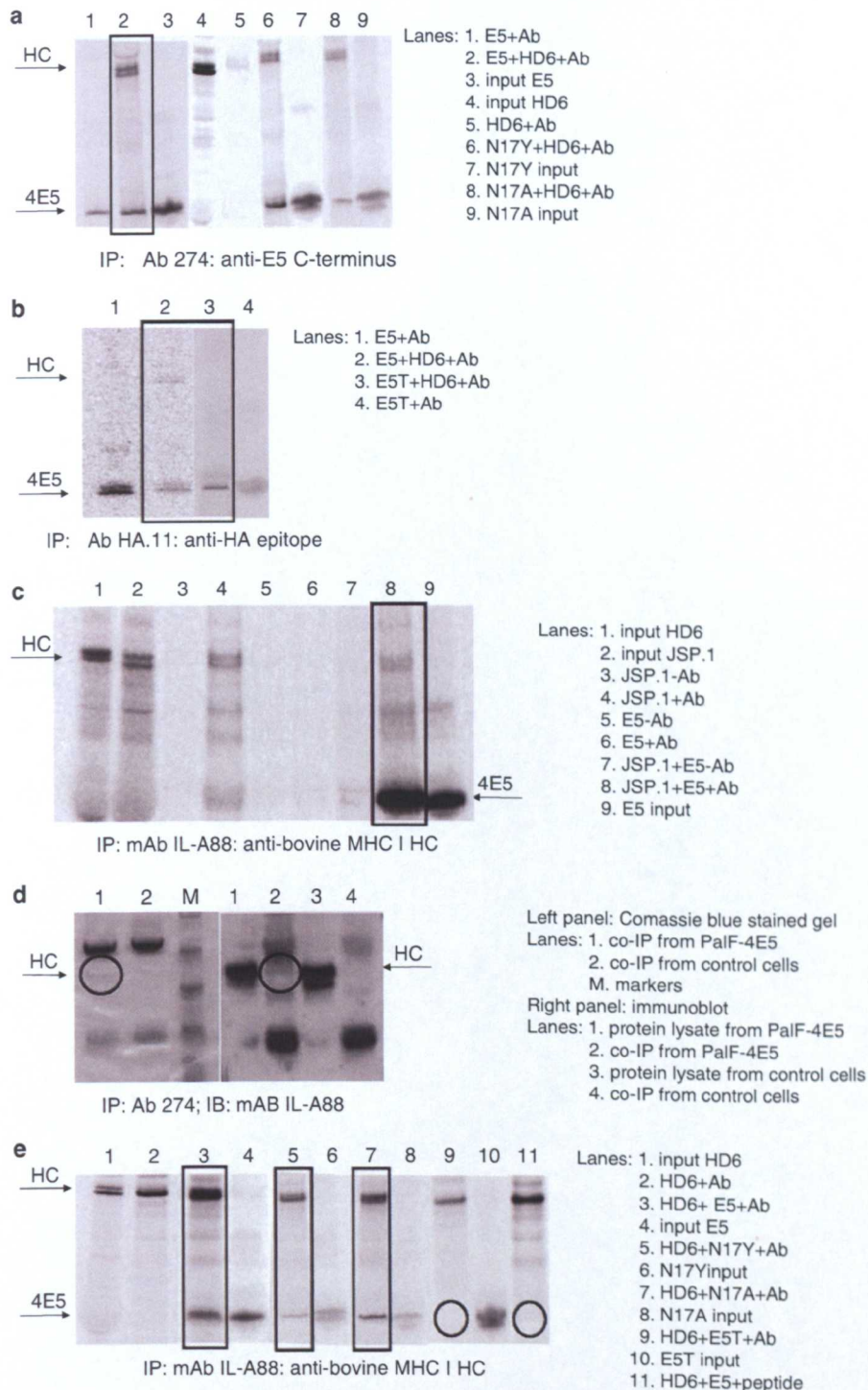
In PalF-4E5 cells, the levels of HC RNA are barely detectable (Ashrafi *et al.*, 2002), and by quantitative RT-PCR we estimate that the amount of HC RNA in these cells is between 10- and 30-fold less than in control cells (Figure 1a, white bars). The reduction of HC RNA is due to a transcriptional inhibition of the HC gene. This conclusion is supported by two observations: E5 downregulates a BoLa HC gene promoter/enhancer (Figure 1b, white bars) and treatment with either β or γ IFN, both of which increase HC gene transcription (Agrawal and Kishore, 2000), leads to accumulation of HC RNA also in PalF-4E5 cells, and to increased activity of the BoLa HC promoter/enhancer (Figure 1, grey and black bars). However, even in the presence of IFN, the levels of HC RNA in PalF-4E5 cells are still approximately 2- to 3-fold lower than in control cells. This suggests that E5 expression causes a transcription inhibitor to bind to the BoLa promoter/enhancer, and that this putative inhibitor exerts its action even when IFN-induced transcription activators promote transcription. This hypothesis however requires confirmation and other explanations are possible. BPV E5 is not present in the nucleus (Burkhardt *et al.*, 1989; Pennie *et al.*, 1993; Zago *et al.*, 2004) and its effect on transcription is therefore not direct. Rather, BPV E5 interferes with several signal transduction pathways (DiMaio and Mattoon, 2001; O'Brien *et al.*, 2001; Zago *et al.*, 2004; Grindlay *et al.*, 2005), and it is therefore likely that inhibition of the BoLa promoter/enhancer is achieved by interference with one or more of these pathways. We have already shown that BPV-4 E5 affects the expression of cyclin A by acting indirectly on the transcriptional promoter of the cyclin A gene (O'Brien *et al.*, 1999; Grindlay *et al.*, 2005) and BPV-4 E5 inhibition of the BoLa promoter/enhancer is another example of E5 interference with transcription regulation.

Degradation of MHC class I HC can be inhibited in PalF cells

MHC class I half-life is regulated by degradation in the proteasomes and lysosomes (Bartee *et al.*, 2004) and several viral proteins contribute to the destruction of MHC class I by favouring HC ubiquitination, or by inducing peptide mis-folding or complex mis-location (Hughes *et al.*, 1997; Hewitt *et al.*, 2002; Bartee *et al.*, 2004). In PalF-4E5 cells, treatment with either proteasome or lysosome inhibitors rescues HC to levels comparable to those of control cells (Figure 2). In 4E5-expressing cells the half-life of HC is drastically reduced (data not shown) and the virtual absence of HC in PalF-4E5 cells is in agreement with the retention of MHC class I in the GA by E5 and with the degradation of mis-folded, mis-located or improperly processed MHC class I by both proteasomes and lysosomes.

BPV E5 interacts with 16k subunit c of the V0 sector of the V-ATPase (Goldstein *et al.*, 1991; Faccini *et al.*, 1996) and this interaction results in alkalization of the GA (Schapiro *et al.*, 2000), which in turn we have argued is in part responsible for retention of MHC class I in the GA (Marchetti *et al.*, 2002). The V-ATPase pump is ubiquitous in the endomembrane compartments, including the lysosomes, so it would have been

expected that its inhibition by E5 would not have lead to HC degradation, or that lysosome inhibitors would have no effect on HC degradation. This apparent discrepancy may be due to different reasons. First, E5 resides mainly in the GA and may inhibit the pump only in this organelle, similar to HPV-16 E5, which alters endosomal pH but not GA pH (Disbrow *et al.*, 2005); second, E5 may disable the pump only transiently



(Ashby *et al.*, 2001), and third, E5 interference with the pump is not complete and stronger inhibition is needed for blocking protein degradation in the lysosomes.

BPV-4 E5 irreversibly blocks transport of MHC class I to the cell surface

The arguments discussed above point to the fact that both E5-induced transcriptional inhibition of the HC

gene and degradation of HC peptide can be overcome. However, the transport of MHC class I from the endomembranes to the cell surface is irreversibly compromised by E5. Although after treatment with IFN and proteasome/lysosome inhibitors, the amount of HC in PalF-4E5 cells is similar to that of control cells, the increase in surface MHC class I is negligible (Figure 3). The alkalization of the GA by E5 is in part responsible for the intracellular retention of MHC class I, as it is also observed in cells treated with the ionophore monensin, which disperses the proton gradient of the GA (Marchetti *et al.*, 2002). However, we found it surprising that treatment with lysosome inhibitors resulted in a large increase in surface MHC class I in control cells but not in PalF-4E5 cells (Figure 3), and hypothesized that an additional reason(s) would be responsible for the inhibition of MHC class I traffic in PalF-4E5 cells.

BPV-4 E5 interacts physically with HC

BPV-1 E5 interacts physically with the receptor for PDGF (Petti and DiMaio, 1994) and both BPV-1 and BPV-4 E5 interact with 16k subunit c (Goldstein *et al.*, 1991; Faccini *et al.*, 1996). Given the established interaction between E5 and membrane proteins, we hypothesized that BPV-4 E5 interacts also with MHC class I HC. Indeed, this is the case and the two proteins coprecipitate *in vitro* and *in vivo* (Figure 4). This interaction is not an artefact, as E5 is precipitated by an antibody against HC even when HC is not labelled (not shown), and E5T, a C-terminal truncated version of E5, does not interact with HC (Figure 4). E5 and HC interact also in PalF-4E5 cells as shown by the coimmunoprecipitation of HC with an antibody against E5 only in PalF-4E5 cells and not in control cells. The reason for the low levels of the HC coprecipitated with 4E5 resides in the fact that, although the levels of HC can be boosted many folds by the combined treatment of IFN and proteasome/lysosomes inhibitors, expression of E5 is not affected; E5 is present at barely detectable levels (O'Brien *et al.*, 1999) and is therefore rate-limiting for complex formation with HC. The interaction between E5 and HC reflects a genuine relationship between the two proteins as there is agreement between interaction *in vivo* and *in vitro* and a good correlation between interaction *in vitro* and downregulation of MHC class I *in vivo*. Furthermore,

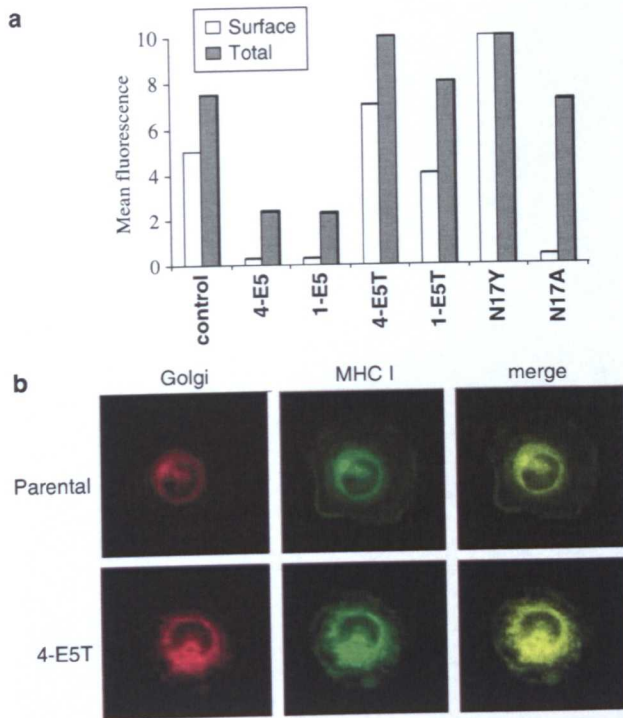


Figure 5 C-terminus deletion mutants of BPV E5 do not downregulate surface MHC class I. (a) The expression of MHC class I in control cells, and in cells expressing wild type or mutant E5 was assessed by flow cytometry analysis with mAb IL-A19. The extent of MHC class I expression is plotted as mean forward fluorescence. (b) Surface localization of MHC class I in parental PalF cells and in cells expressing BPV-4 E5T. MHC class I was detected with mAb IL-A88 and FITC-conjugated secondary antibody (green) and the Golgi apparatus with BODIPY-TR-ceramide (red) in a Leica TCS SP2 confocal scanner microscope. The merge between the FITC and Texas Red fluorescent signals was achieved using the Leica TCS SP2 accompanying software. The nontransforming E5 mutants N17Y and E5T fail to downregulate surface MHC class I.

Figure 4 BPV-4 E5 and MHC class I HC interact via the C-terminus domain of E5. (a) 35 S-labelled *in vitro* transcribed/translated E5, N17Y or N17A, and HD6 HC were immunoprecipitated with Ab 274, against the C-terminus of E5; the precipitate was run in a NuPAGE gel and the dried gel exposed on a screen on a Storm 840 apparatus. Note that lanes 1–3 and lanes 4–9 were run in separate gels and aligned together for ease of comparison. (b) 35 S-labelled *in vitro* transcribed/translated E5 or E5T and HC were immunoprecipitated with mAb HA.11 against the HA epitope tag of E5 and E5T and processed as in (a). E5T does not interact with HD6 HC. (c) 35 S-labelled *in vitro* transcribed/translated E5 and JSP.1 HC were immunoprecipitated with mAb IL-A88 against bovine MHC class I HC and processed as in (a). E5 and JSP.1 HC interact physically. (d) Coimmunoprecipitation of E5 and HC in PalF-4E5 cells. Left panel: Coomassie blue stained gel of protein immunoprecipitates with Ab 274 from PalF-4E5 and PalF control cells. Right panel: immunoblot with mAb IL-A88 of Ab 274-immunoprecipitates from PalF-4E5 and control cells. A band corresponding to HC and reacting with IL-A88 is visible only in the immunoprecipitated from PalF-4E5 cells. (e) 35 S-labelled *in vitro* transcribed/translated E5, N17Y, N17A or E5T and HD6 HC were immunoprecipitated with mAb IL-A88 against bovine MHC class I HC and processed as in (a). E5, N17Y and N17A interact with HC, whereas E5T, deleted in the C-terminus domain, does not. The C-terminus peptide prevents E5 binding to HD6 HC. Note that lanes 5–10 were run in a separate gel and aligned with the other lanes for ease of comparison.

interaction takes place also between HPV-16 E5 and HLA-A in human keratinocytes (Ashrafi *et al.*, in preparation), suggesting that the physical relationship between E5 proteins and HCs is a common mechanism to downregulate surface MHC/HLA class I. We do not know the MHC class I phenotype of the PalF cells but, as the *in vitro* coprecipitation experiments have been done with two different class I alleles, encoded at different loci, and E5 downregulates MHC class I in papillomas from calves of different breeds (Araibi *et al.*, 2004), it is reasonable to conclude that BPV-4 E5 can interact with and downregulate most, if not all classical class I HCs. This is indeed the case for HPV-16 E5 (Ashrafi *et al.*, in preparation).

The interaction between BPV-4 E5 and HC is mediated by the C-terminus domain of E5. The conclusion is based on the three observations that E5T does not inhibit the expression of MHC class I or its transport to the cell surface (Ashrafi *et al.*, 2002), that E5T does not interact with HC and that the interaction between E5 and HC is prevented by a peptide corresponding to the C-terminus of E5 (Figure 4). The C-terminus tail of E5 is clearly critical to the function of the protein: E5T is not transforming (O'Brien *et al.*, 1999), does not induce the typical morphological changes brought about by E5, and does not distort the GA (Ashrafi *et al.*, 2000). The ability to bind MHC class I HC has to be added to the functions of E5 C-terminus. However, the physical interaction between 4E5 and HC appears to be *necessary* but not *sufficient* for downregulation of MHC class I as the E5 mutant N17Y binds HC but does not inhibit MHC class I expression (Figures 4 and 5). This situation is reminiscent of the fact that interaction between E5 and 16k subunit c is not sufficient for cell transformation (Ashrafi *et al.*, 2000; Ashby *et al.*, 2001) and points to a complex interaction of E5 with cellular mechanisms/pathways. Nevertheless, we confirm here our previous observation that only transformation-competent E5 proteins are capable of inhibition of MHC class I.

It is interesting that BPV E5 can potentially interact with more than one membrane protein simultaneously. E5 interacts with 16k subunit c through the transmembrane residue 17 (glutamine in BPV-1 E5, asparagine in BPV-4) (Goldstein *et al.*, 1992b), and could therefore interact with HC through its C-terminus at the same time. BPV-1 E5 interacts with the PDGF receptor through glutamine 17 and the juxtamembrane aspartate 33 (Petti *et al.*, 1997; Klein *et al.*, 1998, 1999) in a multiprotein complex that can include 16k ductin (Goldstein *et al.*, 1992a; Lai *et al.*, 2000). Residue 33 is not present in either 1-E5T or 4-E5T (O'Brien *et al.*, 1999), and we cannot rule out that it plays a role in the interaction with HC. However, although we have not investigated any interaction between BPV-4 E5 and PDGF receptor, cell transformation by BPV-4 E5 does not appear to depend on signalling from growth factor receptors (Zago *et al.*, 2004; Grindlay *et al.*, 2005), and it is therefore unlikely that receptor binding would compete with HC binding.

Other viral proteins, such as HIV Nef (Williams *et al.*, 2002) and HCMV US2 and US8 (Chevalier *et al.*, 2002; Tirabassi and Ploegh, 2002), to name but a few, bind HC and downregulate surface MHC class I, and E5 now joins this large number. It is interesting to point out the parallels between E5 and Nef. Nef binds both Vma13, a component of the V1 sector of the vacuolar ATPase, facilitating the internalization of surface receptors (including MHC class I), and MHC class I HC, disrupting MHC class I traffic (Lu *et al.*, 1998; Williams *et al.*, 2002). Furthermore, as for E5, the same domain of Nef necessary for disruption of MHC class I traffic, mediates the binding to the HC (Williams *et al.*, 2005). Thus, although papillomavirus and HIV are very different viruses, each encodes a protein with similar functions.

Conclusions

We have demonstrated that BPV-4 E5-induced retention of MHC class I in the GA is linked to cell transformation (Ashrafi *et al.*, 2002; O'Brien and Campo, 2003) and is due to the alkalization of the GA (Marchetti *et al.*, 2002). Here we suggest that direct binding between E5 and HC contributes to (but is not sufficient for) the inhibition of MHC class I transport to the cell surface. The expression of E5 is much lower than that of HC, but this stoichiometric imbalance is offset by E5-induced transcriptional inhibition of the HC gene and degradation of the HC peptide. However, E5 prevents traffic of the MHC class I complex even when the amount of HC is greatly increased by drug treatment, therefore, for E5 to exert any effect on HC, the binding must be dynamic. We propose the following hypothesis, consistent with our results: E5 retains newly assembled MHC class I complex in the GA by preventing GA acidification and by physically interacting with HC; the mis-located complex is shunted to lysosomes for degradation, and E5 is free to interact with new MHC class I and re-start the process. While aspects of this hypothesis need confirming, the absence of MHC class I in the E5-expressing cells of naturally occurring BPV-4 induced papillomas supports our suggestion that E5 plays an important role in the establishment and persistence of viral infection by allowing the infect cells to escape host immunosurveillance (Araibi *et al.*, 2004).

Materials and methods

Cells

The bovine foetal PalF cells and their transformed derivatives have been described before (Pennie *et al.*, 1993). Briefly, they are as follows: 'parental' PalF cells are normal foetal primary cells; 'control' cells are cells transformed by BPV-4 E7, HPV-16 E6 and activated ras; PalF-1E5 or PalF-4E5 are 'control' cells additionally expressing either BPV-1 E5 or BPV-4 E5, respectively. The latter cells are referred to as PalF E5 cells when a particular description applies to both PalF-1E5 and PalF-4E5. PalF-N17Y and -N17A express a nontransforming and a hypertransforming mutant of BPV-4 E5, respectively (O'Brien *et al.*, 1999). All the clones analysed within each type exhibit the same characteristics (Pennie *et al.*, 1993; O'Brien

et al., 1999; Ashrafi *et al.*, 2002; Marchetti *et al.*, 2002). Cells were grown in DMEM, 10% foetal calf serum (FCS) at 37°C in 5% CO₂ as previously described (Ashrafi *et al.*, 2002).

Plasmids

pcDNA3.1(-)/HA4E5 contains the BPV-4 E5 ORF (nt 332–460) tagged at the 5' end with the sequence for the HA epitope inserted into the *Bam*HI/*Hind*III sites of pcDNA3.1(-) (Invitrogen, UK). pcDNA 3.1(-)/4E5T contains the HA-tagged BPV-4 E5 ORF in which a premature stop codon has been introduced to terminate the protein at residue 32 (O'Brien *et al.*, 1999). pcDNA3-HD6 and pcDNA-JSP.1 contain cDNA for 2 *Bos taurus* MHC class I HC alleles, HD6 and JSP.1 (GenBank Accession Numbers X80934 and X92870, respectively) (Ellis *et al.*, 1996; Pichowski *et al.*, 1996) inserted in the *Hind*III/*Eco*RI sites of pcDNA3 (Invitrogen, UK). Both pcDNA 3.1(-) and pcDNA3 contain the cloned gene of interest under the control of the T7 promoter for *in vitro* transcription/translation experiments (see below).

pBoLa-Luc was derived from pBoLa-19, a plasmid containing the promoter/enhancer, exon 1 and the 3'UTR of another cattle MHC class I allele (Sawhney *et al.*, 1995, 1996). The promoter sequences were amplified by PCR using the forward primer 5'GTTGAAGGCTCTCGAGGGCATCGGTCGAC3' and the reverse primer 5'TGCAAAGCTTCCTCTGGGTCTGGGAAGAAGC3'. The resulting amplicon of 1100 base pairs was digested with *Xho*I and *Hind*III and inserted between the *Xho*I and *Hind*III sites of pGL3 ahead of the luciferase gene sequences. pBoLa-19 was a kind gift from Dr G Russell.

Treatment of cells with IFN

10⁶ parental, control and PalF-4E5 were seeded in tissue culture flasks. The following day, the medium was replaced with fresh medium with or without 500 U/ml β IFN (Sigma, UK) or 250 U/ml recombinant ovine γ IFN (Graham *et al.*, 1995) for 48 h. Ovine recombinant γ IFN was a kind gift of Dr G Entrican (Moredun Research Institute, Penicuik, UK).

Treatment of cells with lysosome and proteasome inhibitors

Cells were treated either with the lysosome inhibitors Bafilomycin A1 (1 μ M) (Calbiochem, UK) or NH₄Cl (40 mM) (Calbiochem, UK) for 24 h, or with the proteasome inhibitors MG-132 (5 μ M) (Calbiochem, UK) or ALLN (100 μ M) (Merck Biosciences, UK) for 8 h. For combined γ IFN and inhibitors treatment, lysosome inhibitors were added for the last 24 h of γ IFN treatment, and proteasome inhibitors for the last 8 h of γ IFN treatment. The cells were then harvested for detection of MHC class I by immunoblotting or flow cytometry analysis.

Transient transfection and luciferase transcription assays

10⁵ PalF cells were plated in each well of six-well plates, in duplicate, with 5 ml of DMEM, 10% FCS at 37°C in 5% CO₂. After 24 h, the cells were transiently cotransfected with 1 μ g of pBoLa-Luc and 1 μ g of pZipneo or 1 μ g of pZip-4E5 (O'Brien *et al.*, 1999), using the standard Lipofectamine Plus (Invitrogen, UK) method. After 3 h, cells were washed twice with 2 ml PBS and incubated in medium for further 48 h, with or without 250 U/ml γ IFN, before being harvested and lysed in reporter lysis buffer. The lysates were either assayed for reporter enzyme activity immediately or stored at -20°C. Luciferase activity was determined as described previously (O'Brien *et al.*, 1999) and activity was normalized for protein content determined using the BCA assay (Pierce Chemical Co.).

Detection of MHC class I HC RNA by quantitative RT-PCR
Total RNA was extracted from parental, control and PalF-4E5 cells using the RNeasy Mini kit (Qiagen, Sussex, UK), and residual DNA was removed by DNase I treatment according to the manufacturer's guidelines (Invitrogen, UK).

Real-time RT-PCR for MHC I HC and bovine actin mRNA was carried out using the Taqman EZ RT-PCR kit (Applied Biosystems, Foster City, CA, USA) with gene-specific primers and FAM/TAMRA probe designed by primer express v1.7 software. In total, 100 ng RNA was used per each reaction, carried out in triplicate. The primers, spanning exons 2 and 3, were BovMHC_Taq_Forward 5'-TCCGGGCG AACCTGAAC-3' and BovMHC_Taq_Reverse 5'-ACATCT CCTGGAAGGTGTGAGAC-3' and the probe was 5'-CCGCACTCGGCTACTACAACCAGAGC-3'. Bovine actin primers were BovACT_Taq_Forward 5'-CCTCACGGAA CGTGGTTACAG-3' and BovACT_Taq_Reverse 5'-TCTCC TTGATGTCACGCACAA-3' and the probe was 5'-TTACCA CCACAGCCGAGCGGG-3'. RT-PCR was performed using an ABI prism 7700 sequence detector. In each experiment, additional reactions with 10 10-fold serial dilutions of template DNA were performed with each set of primers and probes on the same 96-well plates to generate standard curves. All samples were amplified in triplicate. The relative amounts of MHC I HC and bovine actin mRNA were determined by using the standard curves.

Detection of MHC class I HC by immunoblotting

Cells were removed from the flasks by trypsinization, washed with PBS, then lysed in lysis buffer (100 mM Tris HCl, pH 7.5, 2% SDS, 2% glycerol) and insoluble material was removed by centrifugation at 20 000 g. Lysate (10 μ g) were electrophoresed in 4–12% NuPAGE gels (Invitrogen), and proteins were transferred onto nitrocellulose membrane (Invitrogen) using a semidry blotting apparatus at 20V/150A for 1 h. The membranes were blocked in 5% milk/TBS/Tween-20 (0.5%) at room temperature (RT) for 1 h before incubation with mAb IL-A88 specific for bovine HC (Toye *et al.*, 1990) or mAb AB-1 (Calbiochem) specific for actin. After repeated washing with TBS/Tween-20 (0.5%), the membranes were incubated with anti-mouse Ig-HRP (Amersham Pharmacia Biotech, UK) for mAb IL-A88, and anti-mouse IgM-HRP (Oncogene Calbiochem-Novabiochem International) for mAb AB-1, in 5% milk/TBS/Tween-20 (0.5%) for 1 h at RT. The membranes were washed three times with TBS/Tween-20 (0.5%) and bound antibody was detected by enhanced chemoluminescence staining (ECL) (Amersham Pharmacia Biotech).

Detection of MHC class I by flow cytometry

After removal of the medium, the cells were washed once with PBS, then detached from the flask with trypsin/EDTA and pelleted at 200 g for 5 min at RT. The cell pellet was resuspended in DMEM, 10% FCS, for 1 h at 37°C to allow surface antigens to be re-expressed. The cells were washed and resuspended in PBS, 1% BSA (PBS-B) at 10⁶ cells/ml.

For the detection of surface MHC class I, 100 μ l of cells were aliquoted and incubated with an equal volume of mAb IL-A19 (Bensaid *et al.*, 1989) for 30 min at 4°C. The cells were washed three times in PBS-B and incubated with anti-mouse IgG-FITC (Sigma) at 4°C for 30 min in the dark. The cells were washed and resuspended in 500 μ l PBS-B and analysed by flow cytometry. If the flow cytometry analysis was not carried out immediately, the cells were resuspended in 500 μ l of 3.4% paraformaldehyde (PFA) in PBS and kept at 4°C. For the detection of intracellular MHC class I, the cells were washed in PBS-B and permeabilized with 0.1% saponin in PBS-B for

30 min at RT. Following a further wash in PBS-B, the permeabilized cells were stained with mAb IL-A19 as described above. All samples were examined in a Beckman Coulter EPICS Elite analyser equipped with an ion argon laser with 15 mV of excitation at 488 nm. The data were analysed using Expo 2 software.

Detection of MHC class I and GA by two-colour immunofluorescence

Cells were grown until 80% confluent in single-well chamber slides. After removal of medium, cells were washed twice with serum-free DMEM, 25 mM HEPES (DMEM-H) and incubated in 200 μ l of 5 μ M BODIPY-TR-ceramide, which localizes to the GA, in DMEM-H for 30 min at 4°C. Cells were then washed with DMEM-H for 30 min at 37°C. After removal of the medium, cells were washed twice with PBS and fixed in fixing solution (19 ml PBS, 1 ml 37% formaldehyde and 0.4 g sucrose) for 10 min at RT. For the detection of endogenous MHC class I, after fixation, cells were washed twice and incubated in permeabilizing solution (19 ml PBS, 1 ml 10% NP40 and 0.4 g sucrose) at RT for 10 min and washed as above, then incubated with mAb IL-A88 for 1 h at RT and washed three times as above. The cells were then incubated with anti-mouse IgG-FITC (Sigma) at 4°C for 1 h in the dark. Following three final washes with PBS, the slides were mounted in Citifluor™ and analysed with a Leica TCS SP2 fluorescence confocal microscope. The merge between FITC and BODIPY-TR-ceramide fluorescent signals was achieved with the Leica TCS SP2 accompanying software.

In vitro transcription/translation and coimmunoprecipitation

In vitro transcription/translation reactions were performed using the TNT[®] T7 Quick Coupled Transcription/Translation System (Promega, UK) in presence of Redivue L-[³⁵S]Methionine (Amersham Pharmacia Biotech, UK), following the manufacturer's instructions. Briefly, 1 μ g of pcDNA3.1(-)/HA4E5, pcDNA 3.1(-)/4E5 mutants, pcDNA3-HD6 or pcDNA3-JSP.1 was mixed in a 50 μ l reaction containing TNT[®] mix (TNT[®] lysate with energy generating system, T7 RNA polymerase, nucleotides, salts, recombinant RNasin[®] ribonuclease inhibitors) in presence of canine microsomal membranes (CMM) (Promega, UK) at 30°C for 1.5 h. Half of each transcription/translation reaction product was immunoprecipitated with either 10 μ l rabbit antiserum Ab 274, raised against the C-terminus of the BPV-4 E5 protein (Pennie *et al.*, 1993; Anderson *et al.*, 1997) or with 5 μ l of mAb HA.11

(Cambridge Bioscience, UK) against the HA epitope tagging E5, or with 3 μ l mAb IL-A88. The other half of each reaction was left without antibody as a negative control. For coimmunoprecipitation experiments, the individual transcription/translation products were mixed in equivalent amounts and immunoprecipitated with double the amount of either antibody. For competition experiments, 10 μ g of a synthetic peptide corresponding to the C-terminal 12 amino-acid residues of E5 (Anderson *et al.*, 1997; Araibi *et al.*, 2004) were added to labelled HD6 for an hour before the addition of labelled E5 as above. After incubation overnight at 4°C, protein G-sepharose bead suspension (Sigma, UK) was added for 1 h at 4°C. Following two washes in a high salt buffer (50 mM Tris HCl pH 7.5, 500 mM NaCl, 1% NonidetP-40, 0.05% NaDoc) and one wash in a low salt buffer (50 mM Tris HCl pH 7.5, 1% NonidetP-40, 0.05% NaDoc), the sepharose beads were resuspended in 20 μ l of SDS loading buffer, heated at 75°C for 10 min, and then were electrophoresed in 4–12% NuPAGE gels (Invitrogen). Gels were fixed with glacial acetic acid and methanol, incubated for 15 min in Amplify™ Fluorographic reagent (Amersham, UK), dried and exposed for autoradiography at -70°C overnight or exposed on a screen for quantification on a Storm 840 apparatus using a ImageQuant v5.2 software.

In vivo coimmunoprecipitation

Control PalF cells and PalF-4E5 cells were treated with IFN γ and MG132 as described above. Cells were lysed in RIPA buffer containing a cocktail of protease inhibitors (Roche, Lewes, UK). Protein lysate (100 μ g) were immunoprecipitated with Ab 274 as described above. Immunoprecipitate (10 μ g) were run in NuPAGE gels, transferred onto nitrocellulose membranes and immunoblotted with mAb IL-A88 as described above. A parallel gel was stained with Coomassie blue.

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Appendix (g)

The E5 oncoprotein of BPV-4 does not interfere with the biosynthetic pathway of non-classical MHC class I

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Abstract

The major histocompatibility complex (MHC) class I region in mammals contains both classical and non-classical MHC class I genes. Classical MHC class I molecules present antigenic peptides to cytotoxic T lymphocytes, whereas non-classical MHC class I molecules have a variety of functions. Both classical and non-classical MHC molecules interact with natural killer cell receptors and may under some circumstances prevent cell death by natural killer cytotoxicity. The E5 oncoprotein of BPV-4 down-regulates the expression of classical MHC class I on the cell surface and retains the complex in the Golgi apparatus. The inhibition of classical MHC class I to the cell surface results from both the impaired acidification of the Golgi, due to the interaction of E5 with subunit c of the H⁺ V-ATPase, and to the physical binding of E5 to the heavy chain of MHC class I. Despite the profound effect of E5 on classical MHC class I, E5 does not retain a non-classical MHC class I in the Golgi, does not inhibit its transport to the cell surface and does not bind its heavy chain. We conclude that, as is the case for HPV-16 E5, BPV-4 E5 does not down-regulate certain non-classical MHC class I, potentially providing a mechanism for the escape of the infected cell from attack by both cytotoxic T lymphocytes and NK cells.

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Keywords: BPV; E5; Classical MHC class I; Non-classical MHC class I; E5-MHC class I interaction; MHC class I down-regulation

Introduction

Viruses have to evade the innate and adaptive host immune response to establish infection and persist at least until new progeny virus is released. Many viruses have developed the ability of down-regulating the major histocompatibility complex (MHC) class I (Piguet, 2005), thus avoiding lysis by cytotoxic T lymphocytes (CTL; effector cells capable of recognizing and destroying transformed or infected cells), while not interfering with expression of some non-classical MHC class I genes. Some non-classical MHC class I molecules, in common with classical class I molecules, engage both inhibitory and activating receptors of natural killer (NK) cells, components of the innate immune response, which may

recognize and destroy cells with low levels of MHC class I. The absence of down-regulation of some non-classical MHC class I genes, or their up-regulation, by virus may result in the failure of NK cells to kill virus-infected cells and allows virus to escape both CTL and NK killing (Cohen et al., 1999; Tomasec et al., 2000; Wang et al., 2002).

Papillomaviruses (PV) are small oncogenic viruses that infect mucosal and cutaneous epithelia causing benign hyperproliferative lesions (papillomas, warts, condylomas).

Infection by PV is under immunological control, this is demonstrated by epidemiological studies showing that, in both human and animal hosts with genetic, iatrogenic or acquired cell-mediated immune deficiencies, PV lesions show increased persistence and enhanced neoplastic progression (see O'Brien and Campo, 2002 for review). However, even in immunocompetent individuals, despite expressing abundant viral protein, PV persist for a significant period of time, usually spanning

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several months, and even years, before activation of the host immune system. This lack of recognition suggests that the host immune system is unaware of, and/or disabled by, PV infection. Indeed, the ability of PV to persist has been attributed to several causes: the nature of the virus life cycle, which takes place wholly in the epithelium and therefore prevents high levels of the viral proteins having access to the immune cells; the entry of PV to a latent state with very low levels of expression of viral antigens; and, as demonstrated recently, the ability of the virus to directly subvert and evade the host immune response (Frazer et al., 1999; O'Brien and Campo, 2002; Tindle, 2002). It is likely that the contribution of each of these factors results in a delay or prevention of resolution of infection.

It is becoming clear that the oncoproteins of PV can interfere with the host immunosurveillance and response (O'Brien and Campo, 2002). We have shown that the E5 oncoprotein of both BPV and HPV inhibits the transport of MHC class I to the cell surface and retains it in the Golgi apparatus (Araibi et al., 2004; Ashrafi et al., 2002, 2005; Marchetti et al., 2002). Additionally, we have shown that BPV-4 E5 interacts physically with bovine MHC class I heavy chain (HC) and that both this interaction and the down-regulation of the complex require the C-terminus domain of E5 (Marchetti et al., 2006).

Although effectively down-regulating surface classical MHC class I, HPV-16 E5 does not interfere with the transport of non-classical MHC class I to the cell surface (Ashrafi et al., 2005). It is not known if this is the case also for BPV E5.

Recently, nine putative non-classical class I sequences (*N*50001*–*N*50501*, see <http://www.ebi.ac.uk/ipd/mhc/bola/>) have been identified in cattle cDNAs, most of which have not yet been functionally characterized. *N*50001* (GenBank accession: AY188807) was first identified in 1995 when it was termed HD59 (Ellis et al., 1996) and subsequently was mapped to a region of the cattle MHC containing several classical class I genes (Di Palma et al., 2002). *N*50001* encodes an HC with a truncated cytoplasmic domain (8 rather than 28 amino acids) and in this sense resembles human HLA-G (Geraghty et al., 1987).

Here, we show that BPV-4 E5 cannot down-regulate expression of *N*50001* MHC class I complex or interact with *N*50001* HC, suggesting that, like other viruses, BPV is potentially capable of evading CTL and NK killing of infected cells.

Results

We have shown that BPV-4 E5 binds to bovine class I HC and have mapped the HC-interacting domain of E5 to its C-terminus end (Marchetti et al., 2006). We have also shown that HPV-16 E5 down-regulates classical HLA I (MHC class I) but not non-classical HLA-E (Ashrafi et al., 2005). To determine whether BPV-4 E5, like HPV-16 E5, is incapable of down-regulating certain non-classical MHC genes, we took advantage of a recently described gene in the bovine MHC class I cluster, *N*50001* (Di Palma et al., 2002).

*N*50001* has an early stop codon in the sequence encoding the intracytoplasmic domain (producing a C-terminal truncated HC), has a number of base pairs substitutions leading to unusual amino acids (see Fig. 1), has a large deletion in the 3'UTR and significant differences in the promoter region compared to classical class I genes (data not shown). It is prone to alternative splicing, in common with some other non-classical class I genes, and the isoform most often found was used in this study (Fig. 1). This isoform contains a small section of intron 4 resulting in 4 additional amino acids at the end of the alpha 3 domain. *N*50001* is recognized by mAb IL-A88, an mAb that recognizes a monomorphic determinant on all bovine classical MHC class I molecules (Toye et al., 1990) (Fig. 4). mAb IL-A88 was used throughout the present work.

Generation of mouse P815 cells expressing bovine MHC class I

It is not possible to analyze the expression or the function of *N*50001* in bovine cells (in vivo) because there is no specific antibody for its protein. To investigate the properties of this gene, its cDNA was introduced into the mouse cell line P815, giving rise to P815-*N*50001* cells. P815-*N*01301* cells were generated in a similar way. There was no need to co-transfect the bovine β_2 microglobulin (β_2m) gene with the bovine MHC HC genes into P815 cells because the bovine class I HC associates with mouse β_2m , and when the complex reaches the cell surface, it rapidly exchanges mouse β_2m with bovine β_2m provided by the fetal calf serum in the culture medium (Ellis et al., 2005). Both *N*01301* and *N*50001* are expressed in P815 cells and transported to the cell surface (see below and Fig. 3).

Stable introduction and expression of BPV-4 E5 into P815-*N*01301* and P815-*N*50001* cells

Any interaction between E5 and *N*50001* cannot be studied in bovine cells, such as our well-characterized PalF cell lines as there are not specific antibodies for this HC and the mAb IL-A88, which recognizes *N*50001*, recognizes all other MHC class I HC. The P815-*N*01301* and P815-*N*50001* cells gave us an opportunity to analyze the functional relationship between E5 and *N*50001* independently from the interaction between E5 and *N*01301*. We introduced E5 into these cells, either expressed from the murine MuLV transcriptional promoter in pZipneo (pz4E5) or expressed from the IE promoter of HCMV in pcDNA3 (pc4E5). The corresponding empty vectors were also introduced into the P815-*N*01301* and P815-*N*50001* cells as controls. We generated the following cell lines: P815-*N*01301*-pz4E5, P815-*N*01301*-pc4E5, P815-*N*01301*-pZip, P815-*N*01301*-pcDNA, P815-*N*50001*-pz4E5, P815-*N*50001*-pc4E5, P815-*N*50001*-pZip and P815-*N*50001*-pcDNA.

We determined the expression of E5 by quantitative RT-PCR. There was little or no difference in E5 expression whether expressed from the MLV LTR or the CMV IE promoter, either in P815-*N*01301* or in P815-*N*50001* cells (Fig. 2).

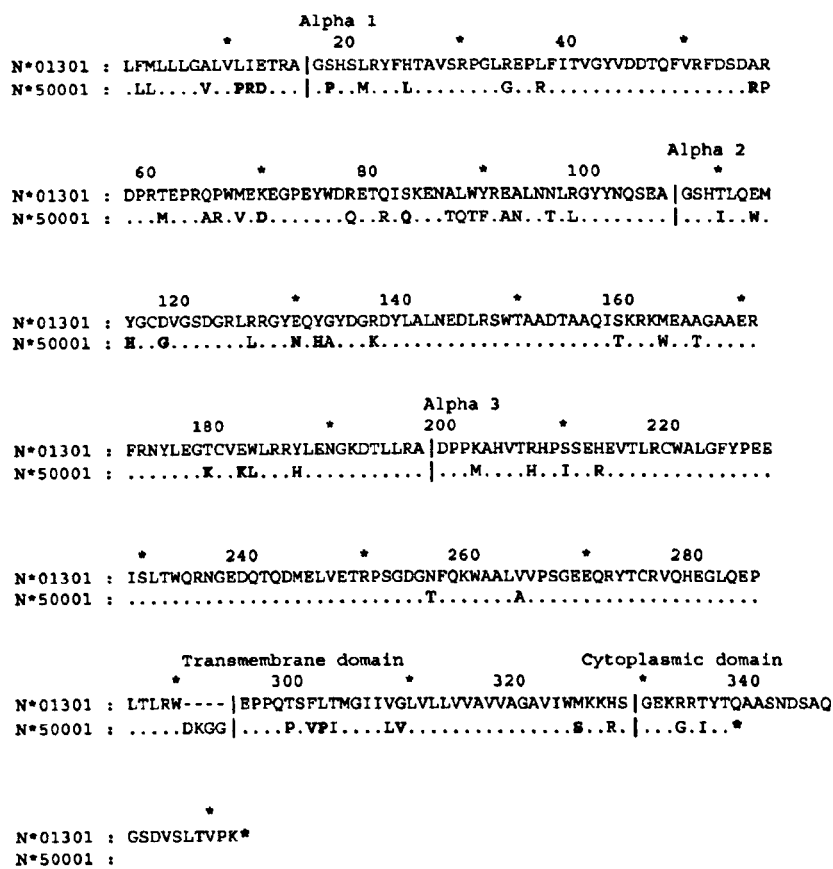


Fig. 1. Alignment of the predicted amino acid sequences of N*01301 and N*50001 (splice variant). Dots indicate identity, *Indicates stop codon. Divergent amino acids (bold) are seen only in N*50001 (or other cattle non-classical class I alleles) and are not seen in cattle classical class I alleles (www.ebi.ac.uk/ipd/mhc/bola/). Four additional amino acids at the end of the alpha 3 domain of N*50001 are the result of incorrect splicing of intron 4.

Expression of bovine MHC class I in P815 cells expressing BPV-4 E5

The expression of N*01301 or N*50001 was analyzed by flow cytometry. As expected, E5 induced a remarkable down-

regulation of N*01301-containing MHC class I (Fig. 3). There was very little surface MHC class I, with a reduction of more than five-fold compared to control cells carrying empty vector, and also the amount of total (surface plus intracytoplasmic) MHC class I was less than half of that in control cells. The

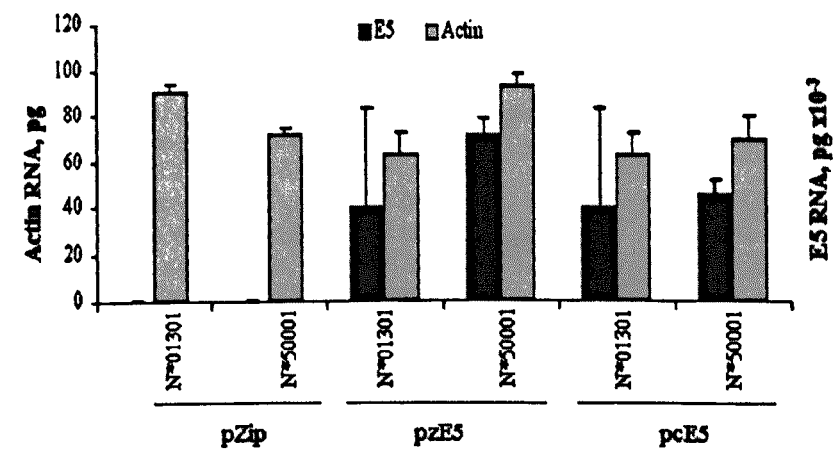


Fig. 2. BPV-4 E5 ORF is transcribed at comparable levels in all P815 cells. E5 and β -actin RNA was measured by Q-RT-PCR. The histograms represent the average with standard deviation of six independent measurements. Note that the levels of E5 RNA are three orders of magnitude lower than those of β -actin RNA.

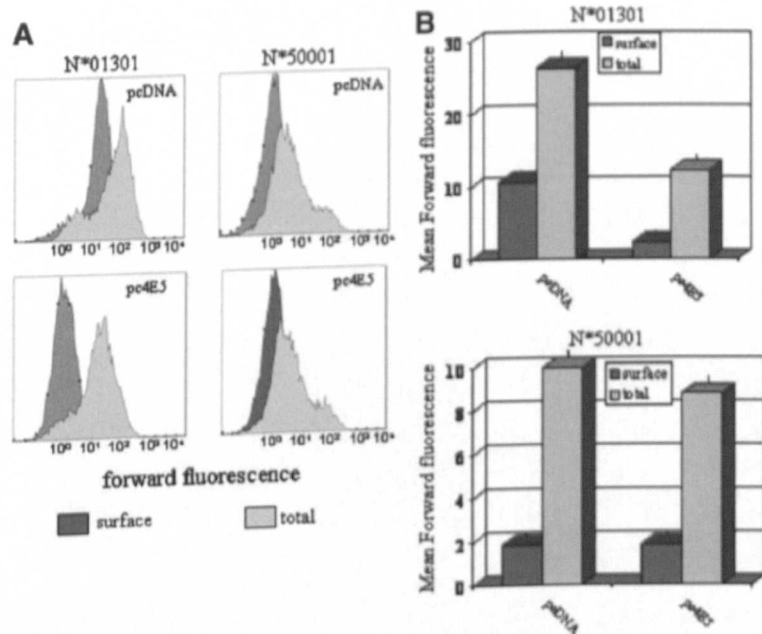


Fig. 3. BPV-4 E5 down-regulates surface and total classical N*01301 MHC class I but not non-classical N*50001 MHC class I. (A) Example of flow cytometry profiles of surface and total classical N*01301 and non-classical N*50001 MHC class I in P815 control cells or cells expressing E5. (B) Mean forward fluorescence with standard deviation calculated from the flow cytometry profiles of at least three independent experiments in duplicate.

results shown in Fig. 3A were obtained with P815-N*01301-pc4E5 cells, but the results obtained with P815-N*01301-pz4E5 cells were essentially identical (data not shown). These results confirm that 4E5 has a drastic effect on the expression and transport of MHC class I (Ashrafi et al., 2002; Marchetti et al., 2006). The results also show conclusively that 4E5 is capable of down-regulating MHC class I in the absence of any other viral oncogene.

In contrast to what was observed with N*01301, N*50001 was not at all affected by E5 and the levels of surface or total MHC were indistinguishable from those in control cells (Fig. 3). Identical results were obtained with P815-N*50001-pz4E5 cells (data not shown). Given the lack of down-regulation of N*50001 by E5 in P815 cells, it is possible that the residual amount of MHC class I detected in bovine cells expressing E5 (Ashrafi et al., 2002; Marchetti et al., 2002) represents non-classical MHC class I expression.

The decrease in N*01301 expression but not in N*50001 expression was confirmed by western blotting. There was less N*01301 expressed in P815-N*01301-pc4E5 or P815-N*01301-pz4E5 cells than in the control cells (Fig. 4A); on the contrary, the expression of N*50001 was unchanged in P815-N*50001-pc4E5 or P815-N*50001-pz4E5 cells, although in control cells it was lower than that of N*01301 (Fig. 4A). It has to be noted that mAb IL-A88 did not react with mouse MHC and class I in the parental P815 cells (Fig. 4A, middle lane), and therefore a possible cross-reaction with bovine and mouse MHC can be discounted. Additionally, mAb IL-A88 did not react with mouse MHC class I in flow cytometry experiments with parental P815 cells (data not shown), confirming lack of cross-reaction.

Turnover of N*01301 and N*50001 in P815-4E5 cells

To investigate the reasons for the decreased expression of N*01301 in the cells expressing 4E5, the half life of the HC was

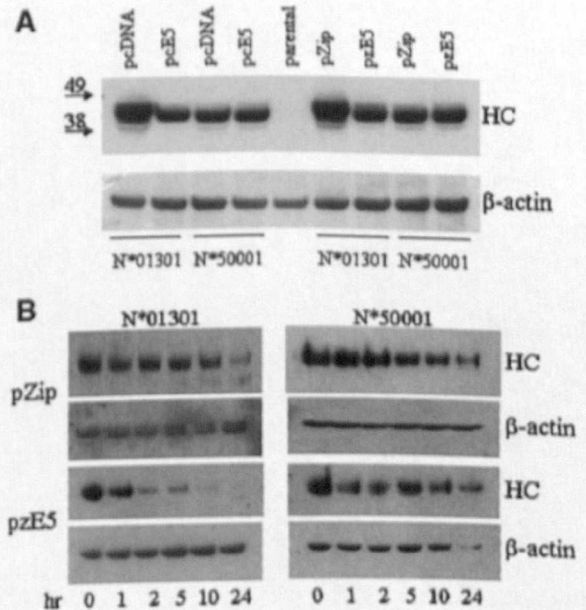


Fig. 4. BPV-4 E5 induces degradation of classical N*01301 MHC class I heavy chain but not non-classical N*50001 heavy chain. (A) Steady state levels of classical N*01301 and non-classical N*50001 heavy chain in P815 control cells or cells expressing E5. (B) Half-life measurement of classical N*01301 and non-classical N*50001 heavy chain in cycloheximide-treated P815 control cells and cells expressing E5. HC, heavy chain.

measured by inhibiting the translation of new proteins with cyclohexamide and by following the turnover of HC by western blotting. In control cells, N*01301 was stable and could still be detected after 24 h of treatment; in remarkable contrast, in the 4E5-expressing cells, N*01301 was unstable and was practically undetectable after 10 h (Fig. 4B). There was no difference between the stability of N*50001 in control or 4E5-expressing cells; in both cases, N*50001 HC was still detectable after 24 h of treatment, comparable to that of N*01301 in control cells (Fig. 4B). These results confirm the general inhibition of classical MHC class I expression by E5, from stability of the HC protein to the transport of the class I complex to the cell surface (Ashrafi et al., 2002, 2005; Marchetti et al., 2002, 2006). The results also show that E5 has no effect on the expression, half-life and transport of non-classical N*50001-MHC class I.

Localization of classical and non-classical MHC class I in P815-4E5 cells

In PalF-4E5 cells, E5 retains the MHC class I in the Golgi apparatus (Marchetti et al., 2002). We wondered whether the reduction in surface N*01301-MHC class I was also due to the retention of residual complex in the Golgi. We performed two-color confocal immunofluorescence in the various P815 cell lines where MHC class I was detected with mAb IL-A88 and an FITC-conjugated secondary antibody and the Golgi apparatus was detected with BODIPY Ceramide Texas Red. In all control cells, whether expressing N*01301 or N*50001, MHC was both associated with the Golgi membranes and clearly on the cell surface (Fig. 5). In contrast, the cellular localization of N*01301- and N*50001-MHC class I was markedly different in P815 cells expressing c4E5 or z4E5. Classical N*01301-MHC class I was totally associated with Golgi membranes, and no complex was detectable on the cell surface with this technique (Fig. 5), whereas, in sharp contrast, N*50001-MHC was clearly on the cell surface, in addition to the Golgi membranes (Fig. 5), and its localization pattern was no different from the one in control cells. Again, no reaction was observed in parental P815 cells (data not shown).

The localization of N*50001 was analyzed also in bovine PalF control cells (transformed but without E5) and in PalF-4E5 cells (transformed and expressing E5) (Ashrafi et al., 2000; Pennie et al., 1993). FLAG-tagged N*50001 was detected both in the Golgi apparatus and on the cell surface in both control and PalF-4E5 cells, while a GFP fusion form of classical HC B2705 was wholly confined to the misshapen Golgi apparatus in the PalF-4E5 cells, but not in the control cells (data not shown), as previously reported (Marchetti et al., 2002). The results obtained in both P815 and PalF cells show beyond doubt that E5 is incapable of inhibiting the transport of N*50001-MHC complex from the Golgi compartment to the surface of the cell.

Physical interaction between E5 and HC

E5 and N*01301 HC interact physically, and we have proposed that this interaction contributes to the down-regulation of the HC and the retention of the complex in the Golgi

(Marchetti et al., 2006). Therefore, we asked whether E5 would complex with N*50001 HC and used in vitro co-precipitation assays as in vitro interaction between E5 and HC is a faithful read-out for in vivo interaction (Marchetti et al., 2006; Ashrafi et al., in press). As before, we in vitro transcribed/translated E5, N*01301 and N*50001 HC and precipitated the HC with mAb IL-A88. Whereas E5 co-precipitated with N*01301 HC, it did not do so with N*50001 HC (Fig. 6A). The lack of interaction between E5 and N*50001 was confirmed by a competition experiment in which unlabeled N*50001 was added to labeled E5 and labeled N*01301. Unlabeled N*50001 failed to prevent the interaction between E5 and N*01301 (Fig. 6A), confirming that it cannot bind to E5.

As described above, N*50001 lacks the C-terminus intracytoplasmic tail of classical MHC class I HC and is 19 amino acids shorter than N*01301 (Fig. 1). Given the absence of interaction between E5 and N*50001, we wondered whether the intracytoplasmic domain of class I HC was responsible for binding to E5. We made a C-terminus truncation mutant of N*01301 HC lacking the final 19 amino acid residues and performed in vitro co-IP experiments with 4E5. This mutant co-precipitated with 4E5 (data not shown), showing that the interaction between HC and 4E5 does not involve the C-terminus intracytoplasmic domain of HC. Furthermore, N*01301 HC bound E5 whether tagged with V5-His at its C-terminus, like N*50001, or not (data not shown), demonstrating that the C-terminus epitope has no effect on the interaction.

We corroborated the in vitro interaction between E5 and HC in a “pull-down co-immunoprecipitation” experiment. Protein lysates for P815 parental cells, P815-N*01301 and P815-N*50001 cells were incubated with labeled E5 and immunoprecipitated with mAb IL-A88. As expected, no E5 could be precipitated from P815 control cells or P815-N*50001 cells, while E5 co-precipitated with N*01301 HC in P815-N*01301 cells (Fig. 6B).

Taken together, the results presented above lead to the firm conclusion that E5 does not interfere with the biosynthetic pathway of non-classical N*50001 MHC class I.

Discussion

Viruses and classical and non-classical MHC class I

It is well-documented that many viruses evade the host immune system by interfering with the antigen processing and presentation pathways (Piguet, 2005). These viral mechanisms often target MHC class I, and there are several examples of discrimination between classical and non-classical class I. While classical class I expression is most often down-regulated by viral interference at one or more points in the pathway between transcription and cell-surface expression, expression of non-classical class I genes usually remains normal (Cohen et al., 1999; Tomasec et al., 2000; Wang et al., 2002). It has been proposed that this enables the virus-infected cell to evade cytotoxic T cell responses while at the same time sending inhibitory signals to NK cells via

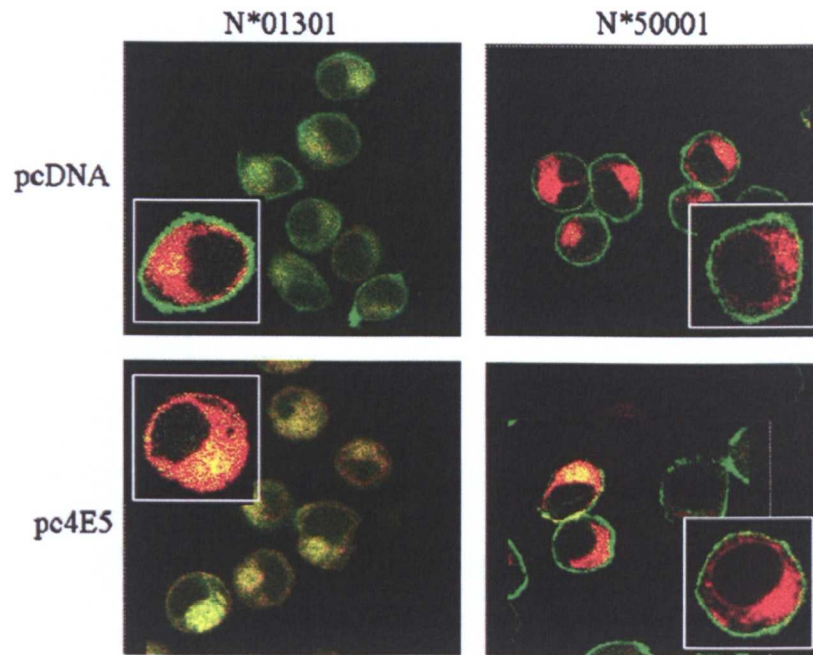


Fig. 5. BPV-4 E5 induces retention of classical, but not non-classical, MHC class I in the Golgi apparatus. (A) Immunofluorescent detection of classical and non-classical MHC class I in P815 control cells or cells expressing E5.

expression of one or more non-classical class I genes. This relates to engagement of inhibitory NK receptors by non-classical class I molecules (Borrego et al., 2005; Hofmeister and Weiss, 2003).

Non-classical MHC class I in cattle

There is considerable evidence that N*50001 and related alleles in cattle are encoded by a non-classical MHC class I

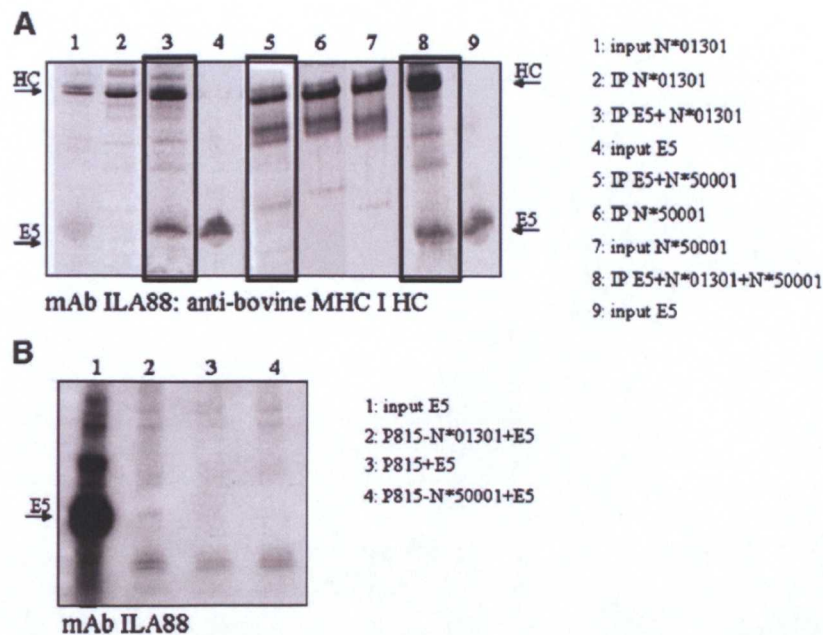


Fig. 6. BPV-4 E5 interacts with classical N*01301 heavy chain but fails to interact with non-classical N*50001 heavy chain. (A) ³⁵S-labeled in vitro transcribed/translated E5 and N*01301 HC or N*50001 HC were immunoprecipitated with mAb ILA88, against MHC class I heavy chain; the precipitate was run in a NuPAGE gel, and the dried gel exposed on a screen on a Storm 840 apparatus. Classical N*01301 HC interacts with E5 (lane 3), while non-classical N*50001 HC fails to interact with E5 (lane 5) and to compete with classical N*01301 HC for binding to E5 (lane 9). Several additional bands are visible which derive from the translation of the proteins (see input lanes) which may be due to dimers or multimers of E5 or to degradation products of HC. (B) “Pull-down co-immunoprecipitation” between E5 and N*01301 HC. Protein extracts from P815, P815-N*01301 and P815-N*50001 cells were incubated with ³⁵S-labeled in vitro transcribed/translated E5 and precipitated with mAb ILA88. E5 is precipitated from P815-N*01301 cells (lane 2) but not from P815 parental or P815-N*50001 cells (lanes 3 and 4).

gene. The classification of *N*50001* (and related alleles) as non-classical is based on several criteria, namely association with a number of unrelated MHC haplotypes (at least one of this set of alleles is present on all MHC haplotypes examined; Ellis, unpublished data), lack of significant polymorphism, a truncated cytoplasmic domain (8 rather than 28 amino acids), unusual amino acid substitutions throughout, a characteristic motif within the transmembrane domain, a large deletion in the 3' UTR and significant differences in the 5' upstream region in comparison to classical class I genes. These alleles may be widely transcribed, and there is currently no evidence to suggest tissue-specific expression (Davies et al., 2004; Ellis, unpublished data). In addition, a number of alternatively spliced transcripts of *N*50001* can be found (Ellis, unpublished data), a characteristic seen in non-classical class I genes in other species, most notably HLA-G (Riteau et al., 2001). *N*50001* and related alleles are transcribed at relatively low levels in peripheral blood lymphocytes and at slightly higher levels in trophoblast tissue during pregnancy (Davies et al., 2004), in common with some other putative non-classical cattle MHC class I alleles.

BPV-4 E5 and MHC class I

Because there is no monoclonal antibody available which exclusively recognizes non-classical class I molecules, to study the relationship of BPV-4 E5 with classical and non-classical MHC I, we separately expressed a classical, *N*01301*, and a non-classical, *N*50001*, allele in mouse P815 cells and subsequently introduced E5 in these cells. This proved to be a powerful system which has confirmed that E5 down-regulates classical MHC class I, while it does not interfere with the biosynthetic pathway of this non-classical MHC class I.

BPV-4 E5 and classical MHC class I

As in bovine PalF cells (Marchetti et al., 2006), E5 down-regulates classical MHC class I at more than one level. However, in PalF cells, E5 was co-expressed with E6, E7 and activated ras (Ashrafi et al., 2000), leaving open the possibility, albeit remote, that down-regulation of MHC class I was due to a functional interaction between the viral and cellular oncoproteins. In P815 cells, E5 profoundly inhibits classical MHC class I in the absence of other viral or cellular oncoproteins. E5 impacts on classical MHC class I at several levels: transcription of the HC gene, expression of protein and transport of the complex to the cell surface (Marchetti et al., 2006). Transcription of the bovine *N*01301* HC cannot be analyzed in P815 cells: E5 inhibits transcription from the homologous promoter of the HC gene, while in P815 cells the *N*01301* HC gene is under the control of the CMV IE promoter, which is not affected by E5 (data not shown). In P815 cells, in addition to blocking MHC class I in the Golgi apparatus, E5 induces the degradation of *N*01301* HC. In control cells, the half-life of the HC is approximately 20 h, whereas in the presence of E5 its half-life is dramatically reduced to less than 5 h. We do not yet know how E5 induces degradation of HC. However, we have commented before (Marchetti et al., 2006) on the similarities between E5

and HIV 1 Nef, and it is interesting to note that Nef re-directs HC to the lysosomes for degradation through the simultaneous interaction with HC and with the adaptor protein 1 (AP-1) complex (Roeth et al., 2004) and that inhibitors of lysosomes (in conjunction with interferon treatment) restore the expression of HC in PalF-4E5 cells (Marchetti et al., 2006). It is thus possible that E5 too interacts with both HC and AP-1 inducing lysosomal degradation of HC, but this remains to be elucidated.

BPV-4 E5 and non-classical MHC class I

In marked contrast to its inhibitory effect on classical MHC class I, E5 does not affect the non-classical MHC class I *N*50001*. This conclusion is based on numerous pieces of evidence. *N*50001* reaches the cell surface, *N*50001* HC is expressed to the same levels as in control cells and its half-life is not affected, and crucially E5 does not interact with *N*50001* HC. This last point is further corroborated by the inability of *N*50001* HC to inhibit the formation of the complex between E5 and *N*01301* HC in vitro. Moreover, the finding that the half-life of *N*50001* HC is not affected by E5 supports our hypothesis that binding to HC is needed for HC degradation.

A major difference between classical *N*01301* and non-classical *N*50001* HC is the truncation of the intracytoplasmic domain in the latter, but the interaction with 4E5 does not take place via this domain. This finding contrasts with the binding of Nef to the intracytoplasmic domain of HLA-A and with the observation that the inability of Nef to bind some non-classical HLA resides in amino acid differences in the intracytoplasmic domain of HLA-E and the absence of the domain in HLA-G (Pizzato et al., 2004; Williams et al., 2002). There are several amino acid differences throughout the extracellular domains between classical *N*01301* and non-classical *N*50001* HC (Fig. 1), but which one of these differences is responsible for the differential interaction with E5 remains to be established.

Biological significance of the lack of down-regulation of non-classical MHC class I by E5

The lack of significant polymorphism in the non-classical MHC class I allele described here strongly suggests that this complex is not involved in antigen presentation and is thus more likely to interact with NK receptors, as has been observed for a number of non-classical class I gene products in human and other species. Cattle have recently been shown to have a family of genes with homology to the human KIRs (McQueen and Parham, 2002; Storset et al., 2003), and several other putative NK receptors have been identified (Storset et al., 2004), but thus far interactions between these putative NK receptors and MHC class I molecules have not been demonstrated.

Thus, while it is possible to speculate that the lack of interaction of E5 with *N*50001* constitutes a viral mechanism for evading NK recognition, this remains to be confirmed. Nevertheless, down-regulation of classical MHC class I and lack of down-regulation of non-classical MHC class I by E5 strongly suggest that the viral protein is instrumental for the establishment of viral infection by allowing the infected cell to

escape both the adaptive and innate immune response of the host.

Materials and methods

Plasmids

N*01301 cDNA, encoding heavy chain (HC) N*01301 (formerly HD6), was cloned both in pcDNA3 (Ellis et al., 1999) and pcDNA6-V5-His (Invitrogen, Paisley, UK). Full-length clones of N*50001 cDNA (AY188807), encoding N*50001, were generated by PCR from cattle PBMC cDNA. This class I gene is very prone to alternative splicing, and the most common splice variant was used in this study. Multiple clones were sequenced, and a clone containing the consensus sequence was introduced into pcDNA6-V5-His (Fig. 1).

pz4E5 and pc4E5 are plasmids carrying the ORF of BPV-4 E5 under the transcriptional control of the murine MLV LTR or the IE promoter of HCMV respectively. These plasmids have been described before (Ashrafi et al., 2002; Pennie et al., 1993).

Cells

P815 cells are a mouse mastocytoma cell line (ATCC). They are grown in suspension in RPMI 1640 supplemented with 10% FCS. Stable transfectants expressing N*01301 were produced by transfection of pcDNA6-V5-His N*01301 and maintained under blasticidin selection. These cells are designed P815-N*01301. Stable P815 transfectants expressing N*50001 were generated as described before (Ellis et al., 1999), and positive cells were identified using blasticidin selection, screening by western blotting with an anti-V5 monoclonal antibody (mAb) (Invitrogen) and flow cytometry. These cells are designated P815-N*50001. Both P815-N*01301 and P815-N*50001 were transfected with either pz4E5 or pc4E5, or the corresponding empty vectors, with an Amara nucleofector (Amara GmbH) using the kits and following the instructions supplied by the manufacturers. Cells were selected by G418 resistance, and stable transfectants were isolated.

Quantitative RT-PCR (Q-RT-PCR)

RNA was extracted from P815 cells using RNeasy mini Kit (Qiagen, UK), and residual DNA was inactivated using DNase I treatment (Invitrogen). Q-RT-PCR for BPV-4 E5 and mouse β -actin mRNA was carried out using the Taqman EZ RT-PCR Kit (Applied Biosystems, Foster City, CA). Each reaction was performed in triplicate using 100 ng of RNA. Oligonucleotide primers, designed using Primer Express (v 1.7, Perkin-Elmer, Oak Brook, IL), were as follows: BPV-4 E5 F5'-TGTCT-TTGTGGCTTATCTATGTTTGT-3'; BPV-4 E5 R5'-CCGAG-TAATAGTAGAAATTAACAGAAGGTACAC-3'; and FAM/TAMRA probe 5'-CTTTTCTGGTGTGCTTTTAATTTTCTTGCACTGTTA-3'. β -actin F5'-GCTCTGGCTCCTAGCAC-CAT-3'; β -actin R5'-GCCACCGATCCACACAGAGT-3' and

FAM/TAMRA probe 5'-AAGATCATTGCTCCTCCTGAGC-GAAAG-3'.

PCR reactions were performed using an ABI prism 7700 sequencer. Standard curves were generated using 10-fold serial dilutions of each template DNA, which were used for quantifying the relative levels of E5 and β -actin RNA.

Flow cytometric analysis of MHC class I expression

P815-N*01301 and P815-N*50001 cells either expressing BPV-4 E5 or carrying empty vector were grown in suspension in T175 cm² flasks until subconfluent. Following centrifugation, the cell pellet was washed with PBS and resuspended in PBS/1% bovine serum albumin (PBS-B) at a concentration of 10⁶ cells/ml. For the detection of surface MHC class I molecules, aliquot of 10⁶ cells was incubated for 1 h at 4 °C with monoclonal antibody (mAb) IL-A88, specific for bovine MHC class I (Toye et al., 1990) in PBS-B. After washing twice with PBS, cells were incubated with 1:100 dilution of anti-mouse IgG-FITC (Sigma, UK) for 30 min at 4 °C in the dark. The cells were washed as above, resuspended in 500 μ l PBS and analyzed by flow cytometry. If the flow cytometry analysis was not carried out immediately, the cells were resuspended in 500 μ l of 3% paraformaldehyde in PBS and kept at 4 °C. P815 cells not expressing bovine MHC class I were used as control, as well as cells incubated without primary antibody. For the detection of intracellular MHC class I, the cells were permeabilized with 0.1% saponin in PBS-B for 30 min at RT and then incubated with primary antibody as described above. All samples were examined in a Beckman coulter EPICS Elite analyzer equipped with an ion argon laser with 15 mV of excitation at 488 nm. Data were analyzed using Expo 2 software.

Localization of MHC class I in P815 cells by immunofluorescence

An eight-well slide (Chamber Slides Lab-tek, VWR International) was washed with alcohol, air-dried and treated with 40 μ l/well poly-L lysine hydrobromide (Sigma) for 10 min to allow adhesion of the cells. After removal of poly-L lysine, the slide was washed with distilled water and air-dried. P815 cells were pelleted, washed twice in PBS and resuspended in fixing solution (5% formaldehyde in PBS containing 2% sucrose) for 10 min. After two washes, the cells were plated in PBS containing 1% FCS, at 1 \times 10² cells/well, and left for 15 min to settle down. After removal of PBS, the cells were treated with permeabilizing solution (0.01% Tween-20 in PBS) for 10 min. The Golgi apparatus was visualized with 1:1000 dilution of BODIPY Ceramide Texas Red (TR) (in 2.5% HEPES in serum-free RPMI 1640 medium) for 30 min at 4 °C. BODIPY Ceramide specifically locates to the Golgi apparatus. After removal of BODIPY Ceramide, cells were incubated in 2.5% HEPES in serum-free RPMI 1640 medium for 30 min at 37 °C. For detection of MHC class I, cells were first incubated with IL-A88 antibody for 1 h at RT followed by incubation with 1:1000 dilution of anti-mouse IgG-FITC for 1 h in dark with

two washes in between. The cells were washed three times, and the slide was mounted with a 60 mm coverslip using citifluor.

Imaging

Images were captured using a Leica TCS SP2 true confocal scanner microscope (Leica Microsystems, Heidelberg, Germany) at a wavelength of 488 nm (MHC class I: FITC) or 543 nm (Golgi apparatus: Texas Red). The merge between the fluorescent signals was achieved using the accompanying software.

Immunoblotting

P815 cells were lysed in 300 µl of lysis buffer (0.5% NP40, 50 mM Tris pH 7.8, 150 mM NaCl) containing a protease inhibitor cocktail (Roche, Lewes, UK). Following incubation on ice for 30 min, the cell debris was eliminated by centrifugation and the protein concentration was measured using the BCA/CuSO₄ assay. To determine the stability of classical and non-classical bovine MHC class I heavy chain in P815 cells, aliquots of 2×10^6 cells were treated with 100 µg/ml cyclohexamide (Sigma) or the solvent DMSO (Sigma) for different periods of time at 37 °C. The cells were washed with ice-cold PBS, pelleted and resuspended in 100 µl of lysis buffer as described above.

The protein samples were mixed with 4× NuPAGE SDS sample buffer (40% Glycerol, 500 mM Tris–HCl (pH 6.8), 8% SDS, 0.075% Serva blue G250, 0.025% phenol red) and 10× NuPAGE Reducing Agent (0.5 M DTT). The samples were heated at 70 °C for 10 min before loading in NuPAGE 4–12% Bis–Tris–HCl (pH 6.4) polyacrylamide gels (Invitrogen Ltd). Electrophoresis was performed in MES SDS Running buffer (Invitrogen Ltd) at a constant voltage of 200 V for 60 min.

Separated protein samples were transferred to nitrocellulose membranes by wet electrophoretic transfer using a Xcell II blotting apparatus (Invitrogen Ltd) as per manufacturer's instructions. The blotting was performed at a constant voltage of 30 V for 80 min in 1× NuPAGE transfer buffer containing 10% methanol. Transfer was checked by staining the nitrocellulose membrane with Ponceau S solution (Sigma).

Membranes were blocked in 5% milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (T-TBS) for 1 h at RT or overnight at 4 °C. For detection of MHC class I, the membranes were incubated with mAb IL-A88 in 5% milk in T-TBS for 2 h at RT. After two washes in T-TBS, the membranes were incubated with 1:5000 dilution of horseradish-peroxidase-linked sheep anti-mouse IgG whole antibody (Amersham Biosciences) in 5% milk in T-TBS for 1 h at RT. Bound antibody was detected by incubating the membrane for 5 min at RT with ECL plus western blotting detection system (Amersham Biosciences) according to the manufacturer's instructions. After exposure, bound antibody was removed from the membranes with stripping buffer (100 mM 2-Mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl pH 6.7) at 50 °C for 30 min. After washing in T-TBS, the membranes were blocked in 5% milk in T-TBS for 1 h at RT prior to re-probing with 1:20,000 dilution

of anti-actin antibody (mAb Ab-1; CALBIOCHEM). After incubation with mAb Ab-1, the membranes were washed two times in T-TBS and incubated with anti-mouse IgG linked to horseradish peroxidase as described above.

Co-immunoprecipitation

In vitro transcription/translation and co-immunoprecipitation

In vitro transcription/translation reactions were performed using the TNT T7 Quick Coupled Transcription/Translation System (Promega, UK) in the presence of Redivue L-[³⁵S] Methionine (Amersham Pharmacia Biotech, UK) following the manufacturer's instructions. Briefly, 1 µg of pc4E5, pcDNA3-N*01301 or pcDNA6-V5-His-N*01301, or pcDNA6-V5-His-N*50001 was mixed in a 50 µl reaction containing TNT mix (TNT lysate with energy generating system, T7 RNA polymerase, nucleotides, salts, recombinant RNasin ribonuclease inhibitors) in the presence of canine microsomal membranes (CMM) (Promega, UK) at 30 °C for 1.5 h. Half of each transcription/translation reaction product was immunoprecipitated with either 10 µl rabbit antiserum Ab 274, raised against the C-terminus of the BPV-4 E5 protein (Anderson et al., 1997; Pennie et al., 1993) or with 3 µl mAb IL-A88. The other half of each reaction was left without antibody as a negative control. For co-immunoprecipitation experiments, the individual transcription/translation products were mixed in equivalent amounts and immunoprecipitated with double the amount of either antibody. For competition experiments, no [³⁵S] Methionine was added to the reaction containing pcDNA3-N*50001 and the unlabeled N*50001 was added to labeled E5 for an hour before the addition of labeled N*01301 as above. After incubation overnight at 4 °C, protein G–sepharose bead suspension (Sigma) was added for 1 h at 4 °C. Following two washes in a high salt buffer (50 mM Tris–HCl pH 7.5, 500 mM NaCl, 1% NonidetP-40, 0.05% NaDoc) and one wash in a low salt buffer (50 mM Tris–HCl pH 7.5, 1% NonidetP-40, 0.05% NaDoc), the sepharose beads were resuspended in 20 µl of SDS loading buffer, heated at 75 °C for 10 min, and then were electrophoresed in 4–12% NuPAGE gels (Invitrogen). Gels were fixed with glacial acetic acid and methanol, incubated for 15 min in Amplify Fluorographic reagent (Amersham, UK), dried and exposed for autoradiography at –70 °C overnight or exposed on a screen for quantification on a Storm 840 apparatus using a ImageQuant v5.2 software.

Pull-down co-immunoprecipitation

Protein lysates were obtained from P815, P815-N*01301 and P815-N*50001 cells. Cells were lysed in RIPA buffer containing a cocktail of protease inhibitors (Roche, Lewes, UK). Protein lysates (100 µg) were incubated with in vitro ³⁵S-labeled E5 overnight at 4 °C and immunoprecipitated with mAb IL-A88 as described above. Immunoprecipitates were electrophoresed in 4–12% NuPAGE gels (Invitrogen). Gels were fixed with glacial acetic acid and methanol, incubated for 15 min in Amplify Fluorographic reagent (Amersham, UK), dried and exposed for autoradiography at –70 °C overnight.

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Appendix (h)

E5 protein of human papillomavirus 16 downregulates HLA class I and interacts with the heavy chain *via* its first hydrophobic domain

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Human papillomavirus type 16 E5 protein (HPV-16 E5) is expressed early in papillomavirus infection and is localised primarily in the cell Golgi apparatus (GA) and endoplasmic reticulum. E5 prevents transport of the major histocompatibility class I (MHC I; HLA class I in humans) to the cell surface and retains the complex in the GA. We report that these effects are due, at least in part, to the interaction between E5 and HLA I heavy chain (HC). We also demonstrate that the down-regulation of surface HLA I and interaction with HC are mediated by the first hydrophobic domain of E5. Although E5 downregulates classical HLA selectively as it does not downregulate non-classical HLA, the interaction with the HC of classical HLA I is not specific for a particular haplotype of HLA I. This suggests that E5 can interfere with antigen presentation by most, if not all, classical HLA I haplotypes, with potentially serious consequences as the ability of infected cells to present antigenic peptides to effector T cells would be compromised.

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Key words: HPV; E5; HLA class I; heavy chain; interaction; immunomodulation

Human papillomaviruses (HPVs) infect mucosal and cutaneous epithelia and induce lesions that can persist and progress to cancer. The mildest forms of HPV disease are benign hyperproliferative lesions, known as warts or papillomas. In most cases, these lesions are cleared after several months following activation of the host immune system against viral antigen.¹ However, due to the ability of certain types of HPVs to avoid immune clearance, occasionally, the lesions do not regress and can progress to cancer. This is especially true for HPV type 16, which is involved in the majority of cases of HPV-induced cervical cancers.² The ability of the virus to avoid immune clearance is due to several factors dependent on the virus life cycle, but also on active mechanisms operated by the viral proteins to counteract the host immune attack. Elimination of virally infected cells requires cytotoxic T-lymphocytes (CTL) that can recognise and kill virally infected cells *via* ligation of their receptor to major histocompatibility class I complex (MHC I; HLA I in humans) bound to viral peptides on the surface of infected cells.³

One of the potential effectors of HPV-16 escape from host immunosurveillance is the viral oncoprotein E5.⁴ E5 is a hydrophobic membrane protein 83 amino acids long, possessing 3 well-defined hydrophobic regions. E5 is expressed early in papillomavirus infection in the deep layers of the infected epithelium^{5,6} (Araibi *et al.*, unpublished results) and is localised mainly in the endoplasmic reticulum (ER) and Golgi apparatus (GA) membranes.⁷ We have previously shown that HPV-16 E5 downregulates the expression of surface HLA I by retaining the complex in the GA, and this downregulation is selective, as E5 does not interfere with nonclassical MHC. This may allow the virus to establish itself by avoiding clearance of virus-infected cells by both CTL and natural killer cells.⁴

Here we show that E5 physically interacts with the heavy chain (HC) of HLA I and that interaction and downregulation of surface HLA I are mediated by the first hydrophobic domain of E5.

Material and methods

Construction of HPV-16 E5 mutants

The plasmid pcDNA-Neo (Invitrogen, UK), encoding G418 resistance and containing the universal IE promoter of CMV, was

used to express HPV-16 E5 wild type (wt) or mutant forms, all tagged with the hemagglutinin (HA) epitope YPYDVPDYA at their N-terminus. HPV-16 E5 deletion mutants R79, A54, V36 and R30 (gift of Dr Alonso, Deutsches Krebsforschungszentrum, Heidelberg, Germany) were generated by introducing double stop codons at the respective nucleotide positions of the HPV 16 E5 sequence.⁸ HPV-16 E5 Del1 mutant protein was made by deletion of the first hydrophobic domain (amino acids 1–30) by PCR amplification using forward primer from nt 91 to 105 of the HPV-16 E5 ORF (5'-CATTGCTAGC ATGTACCCAT ACGATGTTCC AGATTACGCT CCGCTGCTTTTGTCT-3') and reverse primer from nt 252 to 231 (5'-TCGCGAATTCTTATGTAATTAATAAAGCGTG-3') including sites for Eco RI and Nhe I respectively. The resulting PCR products were inserted between the Eco RI and Nhe I sites of pcDNA-Neo. All clones were verified by sequencing.

Construction of GFP-E5 fusion proteins

pEGFP-C1 (Clontech, UK) is a eukaryotic expression plasmid, which encodes the green fluorescent protein (GFP). It was used to clone full length HPV-16 E5 and its mutants to generate GFP-E5 wt and GFP-E5 mutant fusion proteins by PCR. The forward primer was from nt 1 to 22 of the E5 ORF for E5 wt and mutants R79, A54, V36 and R30, and from nt 91 to 112 for the Del1 mutant. The reverse primers were from nt 252 to 231 for Del1, from 237 to 216 for R79, from 162 to 141 for A54, from 111 to 90 for V36 and from 93 to 72 for R30. The forward and reverse primers included an Eco RI and a Bgl II site respectively. The resulting PCR products were inserted between the Eco RI and Bgl II sites of pEGFP-C1. All clones were verified by sequencing.

Cell culture and transfection

The immortalised human keratinocyte (HaCaT) cell line was grown in Dulbecco's modified Eagle medium (DMEM) high glucose without calcium chloride (Life Technologies, UK), supplemented with 10% foetal calf serum, as previously described.⁹ Cells (1×10^6) were stably transfected with 4 mg pcDNA, pc-16E5 or pc-16E5 mutants by using Lipofectamine Plus (Invitrogen). Following transfection, HaCaT cells were selected in medium containing 500 µg/ml G418 for 21 days. G418-resistant colonies were marked, picked and expanded into cell lines for analysis.

Real time/quantitative RT-PCR

RNA was isolated from HaCaT cells using the RNAeasy Mini kit (Qiagen, Sussex, UK), and residual DNA was removed by DNAase I treatment according to the manufacturer's guidelines (Invitrogen). Real-time RT-PCR for E5, E5 mutants and β -actin mRNA was carried out using the Taqman EZ RT-PCR kit (Applied Biosystems, Foster City, CA) with gene-specific primers and FAM/TAMRA probe designed by primer express v1.7 software. RNA (100 ng) was used per each reaction, done in triplicate.

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Primers were as follows: forward (F) primer for E5 wt, R79, A54, V36 and R30 was 5'-TGACAAATCT TGATACTGCATCCA-3'; reverse (R) primer for E5, R79 and A54 was 5'-TGACAAATCT TGATACTGCATCCA-3'; R primer for V36 and R30 was 5'-TAATAGGCAG ACACACAAA-3'.

The probe was 5'-AACATTACTG GCGTGCTTTT TGCTTT-GCT-3' in all cases. For β -actin, primers and probe were commercially available (Applied Biosystems). Standard curves were generated using 10-fold serial dilutions of each template DNA, to quantify the relative levels of E5 and β -actin mRNA.

Semi-quantitative RT-PCR

Real time RT-PCR did not work for E5 Del1, as no appropriate forward primer could be identified outside the first hydrophobic domain that matched the probe. Therefore, to quantify the levels of E5 Del1, semi-quantitative RT-PCR was performed.⁹ The Del1 F primer was 5'-CTGCTTTTGT CTGTGTCTACATA-3' and the R primer was 5'-TATAATATAT ACAATAAACACCTAA-3'. RT-PCR was performed using an ABI prism 7700 sequence detector for 20, 25, 30 or 35 cycles. The same amplification protocol was used with E5 wt and R30 as controls. The RT-PCR products were run on gels, the bands scanned with MagicScanner 32-v4.3 and quantified with ImageQuant v5.2 software.

Western blotting

Fifty microgram of protein lysates from HaCaT cell harbouring empty vector (Control), expressing Del1 or E5, were electrophoresed and transferred to nitrocellulose membrane. The membranes were blocked and incubated with mAb HA11 (1/500 dilution; Sigma) for 1 hr, washed and incubated with antimouse IgM-HRP (1/5,000; Oncogene Calbiochem-Novabiochem International). After 1 hr, the membranes were washed and bound antibody was detected by enhanced chemoluminescence staining (Amersham Pharmacia Biotech, UK).

Flow cytometry

Detection of HLA I by flow cytometry was performed essentially as previously described.⁴ Briefly, HaCaT cells and cells expressing E5 and mutants were incubated with antihuman HLA class I monoclonal antibody (mAb) W6/32 (1/100; Serotec) at 4°C for 30 min, and then with antimouse IgG-FITC (1/100; Sigma) at 4°C for 30 min in the dark. After washing, the cells were analysed by flow cytometry. If the flow cytometry analysis was not performed immediately, the cells were re-suspended in 500 ml of 3.4% paraformaldehyde in PBS and kept at 4°C. For the detection of intracellular HLA I, the cells were permeabilised with 0.5% Saponin (Sigma) in PBS-B and incubated with the primary antibody as described earlier. All samples were examined in a Beckman Coulter EPICS Elite analyser equipped with an ion argon laser with 15 mV of excitation at 488 nm. The data were analysed using Expo 2 software.

Immunofluorescence

Detection of HLA I by immunofluorescence was performed essentially as previously described.⁴ Briefly, cells were washed and fixed in fixing solution (19 ml PBS, 1 ml 37% formaldehyde and 0.4 g sucrose) for 10 min at room temperature (RT). After fixation, cells were incubated in permeabilising solution (19 ml PBS, 1 ml 10% NP40 and 0.4 g sucrose) at RT for 10 min, incubated with mAb W6/32 (1:50 dilution) for 1 hr at RT and then with antimouse IgG-FITC (1/500; Sigma) at 4°C for 1 hr in the dark. Following 3 final washes with PBS, the slides were mounted in CitifluorTM (glycerol/PBS solution, Sigma) and analysed with a Leica TCS SP2 fluorescence confocal microscope (Leica-microsystems, Heidelberg, Germany).

Co-localisation of GA and E5

HaCaT cells were transiently transfected with 0.1 μ g of either pEGFP-E5 or pEGFP-E5 mutants, or with control empty plasmid,

using Lipofectamine PlusTM Reagent according to the manufacturer's instructions. Seventy-two hours after transfection, cells were plated approximately 25–50% confluent and grown overnight. Cells were washed twice with serum-free DMEM, 25 mM Hepes (DMEM-H) and incubated in 200 μ l of 5 μ M BODIPY-TR-ceramide, which localises to the GA, in DMEM-H for 30 min at 4°C. Cells were washed twice with PBS and fixed in fixing solution as earlier, and washed 3 times with PBS. Cells were incubated with 4',6'-diamino-2-phenylindole (DAPI) for 10 min, to stain the nucleus, washed in PBS-FCS and then with distilled water, dried and mounted in CitifluorTM (Sigma). Cells were analysed with a Leica TCS SP2 confocal scanner microscope. Images were acquired and merged using Leica confocal software.

Co-immunoprecipitation of E5 and HLA HC in HaCaT cells

Control or E5-HaCaT cells were lysed in RIPA buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% NaDoc, 0.1% SDS) containing a cocktail of protease inhibitors (Roche, Lewes, UK). Protein lysate (100 μ g) was immunoprecipitated with 50 μ l of mAb HA11 (1:10 dilution; Sigma) against the HA-tag of E5 and then incubated at 4°C overnight. After that, protein G-sepharose bead suspension (Sigma) was added for 1 hr at 4°C. Following 4 washes in RIPA buffer, the sepharose beads were resuspended in 20 μ l of SDS loading buffer, heated at 75°C for 10 min and then electrophoresed in 12% NuPAGE gels (Invitrogen). Proteins were transferred onto nitrocellulose membranes and immunoblotted with 10 μ l mAb HC10 against HLA-A, B, C (1:1,000 dilution; Cancer Research, UK).

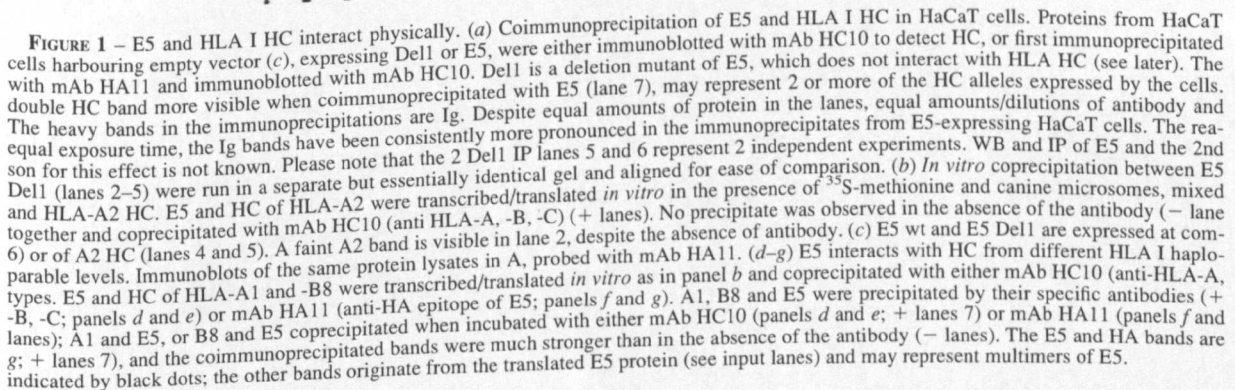
In vitro transcription/translation and coimmunoprecipitation

HLA HC A2 cDNA sequence in pAL356 was excised and re-cloned in pBluescript II SK (+) (Stratagene); HLA HC A1 or B8 cDNA sequences in pRSV5-neo were excised and re-cloned in pGEM-11Zf (Promega, UK). All HLA HC plasmids were a gift from Dr S. Man, University of Cardiff, UK. HPV-16 E5 or E5 mutants and A1, A2 or B8 were *in vitro* transcribed-translated using the TNT[®] T7 Quick Coupled Transcription/Translation System (Promega) in presence of Redivue L-[³⁵S] methionine (Amersham Pharmacia Biotech) following the manufacturer instructions, and as described previously.¹⁰ Half of each transcription/translation reaction product was immunoprecipitated with 3 μ l of mAb HA11 (1:10 dilution; Sigma) or, in the case of R30, 3 μ l of a polyclonal antiserum raised against the N-terminus of E5 (gift of Prof D. DiMaio, Yale University, USA; Hwang et al., 1995), or 10 μ l mAb HC10 against HLA-A, B, C (1:250 dilution; Cancer Research, UK) and then incubated at 4°C overnight. The other half of each reaction was left without antibody as a negative control. For coimmunoprecipitation, the individual transcription/translation products (25 μ l each of HLA HC and E5 reactions) were mixed in equivalent amounts and immunoprecipitated with double the amount of either antibody. For competition experiments, unlabelled R30 or Del1 was added to labelled HLA-A2 overnight before the addition of labelled E5 and antibody as earlier. After incubation overnight at 4°C, protein G-sepharose bead suspension (Sigma) was added for 1 hr at 4°C. Following 2 washes in a high salt buffer (50 mM Tris HCl, pH 7.5, 500 mM NaCl, 1% Nonidet P40, 0.05% NaDoc) and 1 wash in a low salt buffer (50 mM Tris HCl, pH 7.5, 1% NonidetP-400, 0.05% NaDoc), the beads were resuspended in 20 μ l of SDS loading buffer, heated at 75°C for 10 min and electrophoresed in 4–12% NuPAGE gels (Invitrogen). Gels were fixed with glacial acetic acid and methanol, incubated for 15 min in AmplifyTM Fluorographic reagent (Amersham, UK), dried and exposed for autoradiography at –70°C overnight or exposed to a screen in a Storm 840 apparatus using a ImageQuant v5.2 software.

Results

HPV-16 E5 and HLA I HC interact physically

HPV-16 E5 prevents the transport of HLA class I to the cell surface by retaining the complex in the GA.⁴ We have recently



present in the precipitate from E5-expressing cells, while no band corresponding to HC was detected in the precipitate from control cells (Fig. 1a, lanes 4 and 7). In addition to being detected by mAb HC10, specific for HLA class I, the band corresponded to the HC detected in the protein lysate by Western blotting (Fig. 1a, lanes 1 and 2), leading to its identification as HC, and to the conclusion that E5 and HLA-A3 HC (and possibly A31 B60 B51, as suggested by the double HC band) exist in a physical complex in

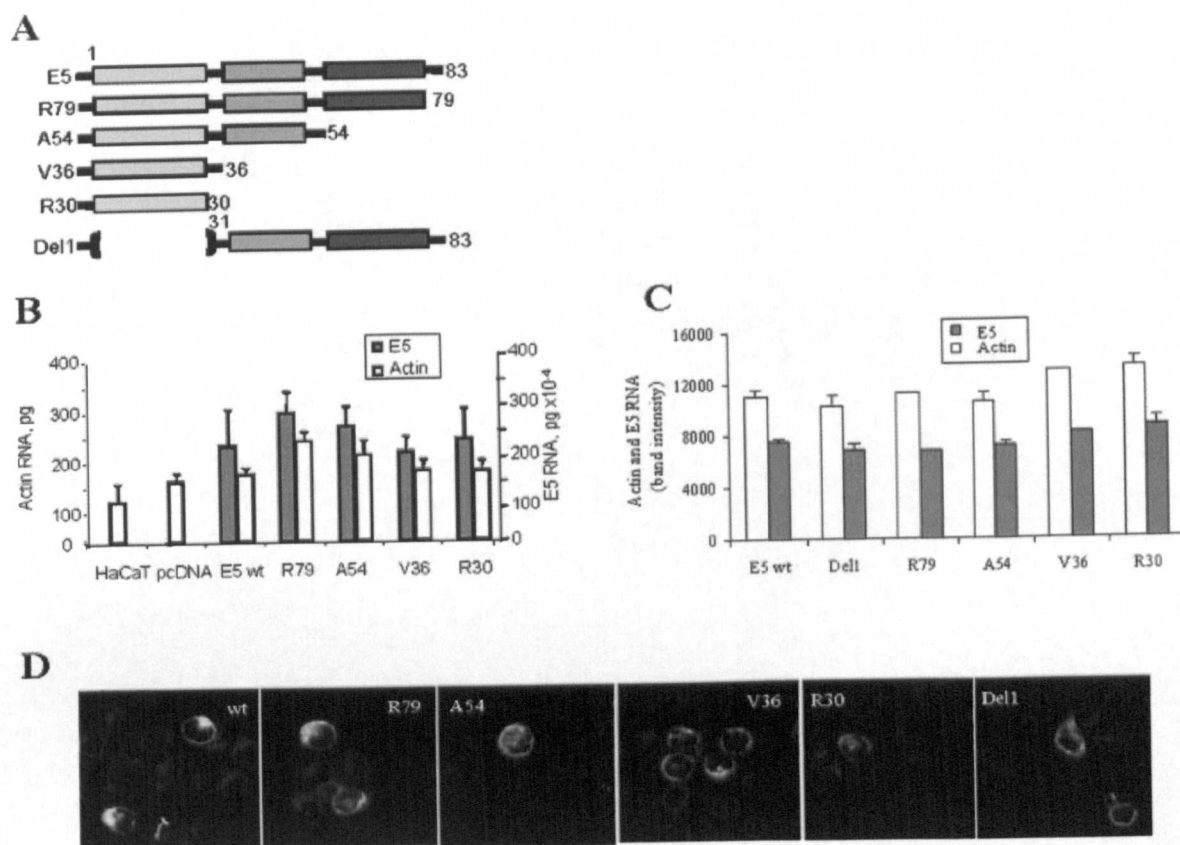


FIGURE 2 – Deletion mutants of E5 express to the same levels and have similar cellular localisation to E5 wt. (a) Diagrammatic representation of E5 wt, showing the 3 hydrophobic domains, and E5 mutants, showing the missing domains of E5; (b) Quantitative RT-PCR for E5 wt, E5 mutant and actin RNA, showing that the E5 mutants express at comparable levels; note that the right-hand y-axis represents the amount of E5 RNA and is 1×10^{-4} that of the left-hand y-axis, which represents the amount of actin RNA; (c) Semi-quantitative RT-PCR for E5 wt, E5 mutant and actin RNA, showing that E5 Del1 mutant express at levels comparable with E5 wt. The y-axis represents the band intensity after 25 amplification cycles as measured by ImageQuant v5.2 software. (d) GFP-fusion forms of E5 wt or E5 mutants, transiently transfected in HaCaT keratinocytes, were visualised at the confocal microscope, along with the GA stained with BODIPY-TR-ceramide and the nucleus stained with DAPI. Only the merged images are presented (in greyscale), which show that E5 wt and mutants localise in the GA and ER. As the HaCaT cells were transiently transfected with the GFP plasmids, GFP-E5 fusion proteins are expressed only in a minority of cells.

E5-expressing HaCaT cells (Del1 is a noninteracting mutant of E5, which is described later). The relative faintness of the HC band coprecipitated with E5 is due to the very low levels of E5 in these cells: E5 is expressed at approximately 4 orders of magnitude lower than actin (see Fig. 2b and Ref. ⁴). Additionally, we performed an *in vitro* coimmunoprecipitation experiment between ³⁵S-labelled E5 and HLA-A2 HC, as a similar experiment had already validated the interaction between BPV-4 E5 and bovine MHC I HC.¹⁰ Physical interaction between E5 and HC was confirmed by the coimmunoprecipitation of ³⁵S-labelled E5 and HLA-A2 with mAb HC-10 (Fig. 1b, lane 7). Moreover, the formation of a complex between E5 and HLA-A2 HC *in vitro* indicates that the interaction is not specific for a particular HLA I haplotype.

E5 interacts with the HC of different HLA I haplotypes

To investigate whether E5 interacts with different HC, and as the *in vitro* interaction between E5 and HC reflects the interaction occurring *in vivo*, we performed *in vitro* coprecipitation experiments with E5 and A1 or B8 HC. In both cases, E5 and HC coprecipitated when either mAb HC10 or mAb HA11 was used (Figs. 1d–1g, lanes 7). Although a faint band corresponding to A1 or B8 HC was detected with mAb HA11 even in the absence of E5, the band was stronger when E5 was present, particularly in the case of B8, giving confidence that the coprecipitation reflects a

genuine interaction. As E5 interacts with A3 in HaCaT cells and with A1, A2 and B8 *in vitro*, we conclude that the interaction between E5 and HC is likely to take part with most, if not all, classic HLA I alleles.

Characterisation of E5 mutants

HPV-16 E5 is a transmembrane protein containing 3 well-defined hydrophobic regions, of which the first one is the longest.^{12,13} To determine the E5 domain responsible for downregulation of surface HLA I, deletion mutants of E5, lacking helical domain 1, 2 or 3, were assayed for their ability to retain HLA I in the GA. Mutant R79 lacks the last 5 C-terminal amino acids, A54 lacks the complete third hydrophobic domain, V36 and R30 lack the second and third hydrophobic domains and Del1 lacks the first hydrophobic domain (Fig. 2a). Before any analysis of the E5 mutants was carried out, we made sure that their expression and cellular localisation were not different from those of E5 wt.

Expression of E5 in stably transfected cells. We verified that the E5 proteins were being transcribed to a similar extent using quantitative RT-PCR (Q-RT-PCR). RNA was isolated from 3 clones each of HaCaT keratinocytes stably transfected with E5 wt or mutants. The level of E5 or E5 mutant RNA (with the exception of E5 Del1, see later) was determined and compared to the level of β -actin RNA. Representative results from 1 clone each of E5

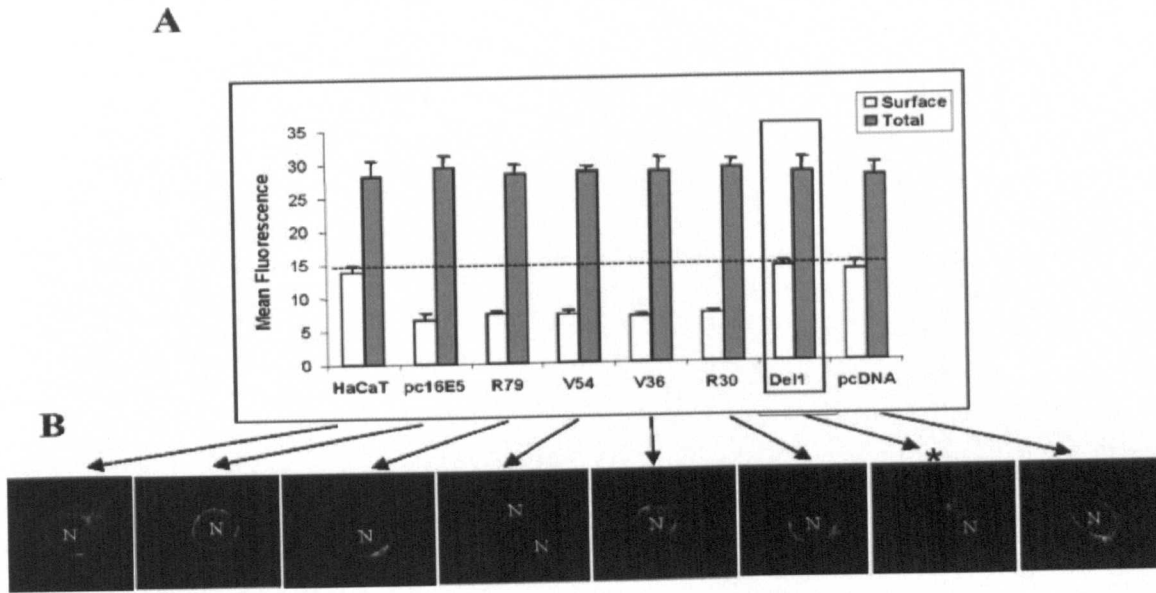


FIGURE 3 – The first hydrophobic domain of E5 mediates the downregulation of surface HLA I. (a), HaCaT parental cells, HaCaT carrying empty vector, or expressing E5 wt or mutants were analysed by flow cytometry for surface (white bars) and total HLA I (grey bars). The average mean fluorescence was calculated from the flow cytometry analyses of 2 duplicate measurements from at least 3 clones of each cell line. The background (the reading of cells stained with no primary antibody and only secondary antibody) was 0.4 in all cases. Standard deviation is shown. Del1 fails to downregulate surface HLA I. The dotted line indicates the level of surface HLA I in parental and control cells. The HLA I values for Del1 are boxed. (b) The same cells were stained with mAb W6/32 and analysed by cytoimmunofluorescence. Del1 mutant failed to retain HLA I in the GA (highlighted with *). Although only a few cells are shown, the localisation of HLA I was the same in all cells of each clone. N, nucleus.

wt and mutants are shown in Figure 2b. The levels of E5 wt or mutant RNA were very low, approximately 4 orders of magnitude less than actin RNA as previously found⁴; however, they were comparable among clones, ranging approximately from 0.02 to 0.03 pg per 100 ng RNA.

Del1 RNA could not be amplified by Q-RT-PCR because no forward primer compatible with the probe could be identified outside the first hydrophobic domain, so semi-quantitative RT-PCR was used instead. Also in this case, there was no appreciable difference in the amounts of RNA between E5 wt and mutants, including Del1 (Fig. 2c).

Cellular localisation of E5 proteins. Given the very low expression levels of E5, it is not possible to determine the cellular location of the protein by cytoimmunofluorescence. Therefore, we decided to analyse E5 location by the use of GFP-E5 fusion proteins. The correct cellular localisation of GFP-E5 fusion proteins has been reported.¹⁴ HaCaT cells were transiently transfected with plasmids encoding GFP-fusions of E5 or E5 mutants or empty EGFP vector; the GA was visualised by using the Golgi marker BODIPY-TR-ceramide and the nucleus was stained with DAPI. The localisation of the GFP-fusion proteins was determined by three-colour confocal microscopy. There was no discernible difference in localisation between E5 and its mutants; in all cases, the E5 proteins were localised in the endomembranes, mainly the GA, as shown by the merged images (Fig. 2d).

The first hydrophobic domain of E5 is responsible for surface HLA I downregulation

As E5 wt and mutants were expressed to comparable levels and localised to similar cellular compartments, we next investigated which E5 domain(s) was responsible for prevention of transport of MHC class I to the cell surface and retention of the complex in the GA. Using flow cytometry, we determined the levels of both surface and total (surface plus intracellular) HLA I in parental HaCaT, control cells harbouring empty vector, cells stably

expressing E5 wt or mutants. Control cells were not different from parental cells, with approximately half as much surface HLA I than total HLA I (Fig. 3a). In clones expressing E5 wt, the levels of surface HLA I were reduced to approximately half that of control cells, as previously reported⁴; likewise, clones expressing the E5 mutants containing the first hydrophobic domain (R79, A54, V36, R30) had reduced levels of surface HLA I to the same extent as E5 wt (Fig. 3a). In contrast, expression of E5 Del1, lacking the first hydrophobic domain, did not have any effect on the levels of surface HLA I (Fig. 3a). The failure of E5 Del1 to downregulate surface HLA I could be due to impaired stability of the protein. However, Western blotting of protein lysates from E5 wt- or E5 Del1-expressing HaCaT cells showed that this is not the case as E5 wt and E5 Del1 were present at comparable levels (Fig. 1c, lanes 1 and 3).

From these results, we conclude that the first N-terminus 30 amino acids of E5 are responsible for the downregulation of HLA I.

This conclusion was confirmed by the localisation of HLA I in HaCaT cells expressing the E5 proteins. Cells carrying empty vector, expressing E5 wt or E5 mutants, were stained with mAb W6/32 and analysed for HLA I by cytoimmunofluorescence. In cells expressing E5 wt or mutants R79, A54, V36 or R30 (all containing the first hydrophobic domain), HLA class I was detected only intracellularly (Fig. 3b); in contrast, in cells expressing E5 Del1 HLA I was detected both on the cell surface and intracellularly, as in control cells (Fig. 3b). These results, together with those obtained by flow cytometry, confirm that E5 amino acids 1–30 are responsible for the retention of HLA class I complex in the GA.

HPV-16 E5 binds HLA I HC via its first helical domain

E5 interacts with HLA I HC (Fig. 1) and prevents the transport of HLA I to the cell surface via its first hydrophobic domain (Fig. 3). To investigate whether the same E5 domain was responsible for the interaction with HC, we performed *in vivo* and *in vitro* coimmunoprecipitation experiments as earlier. In HaCaT cells

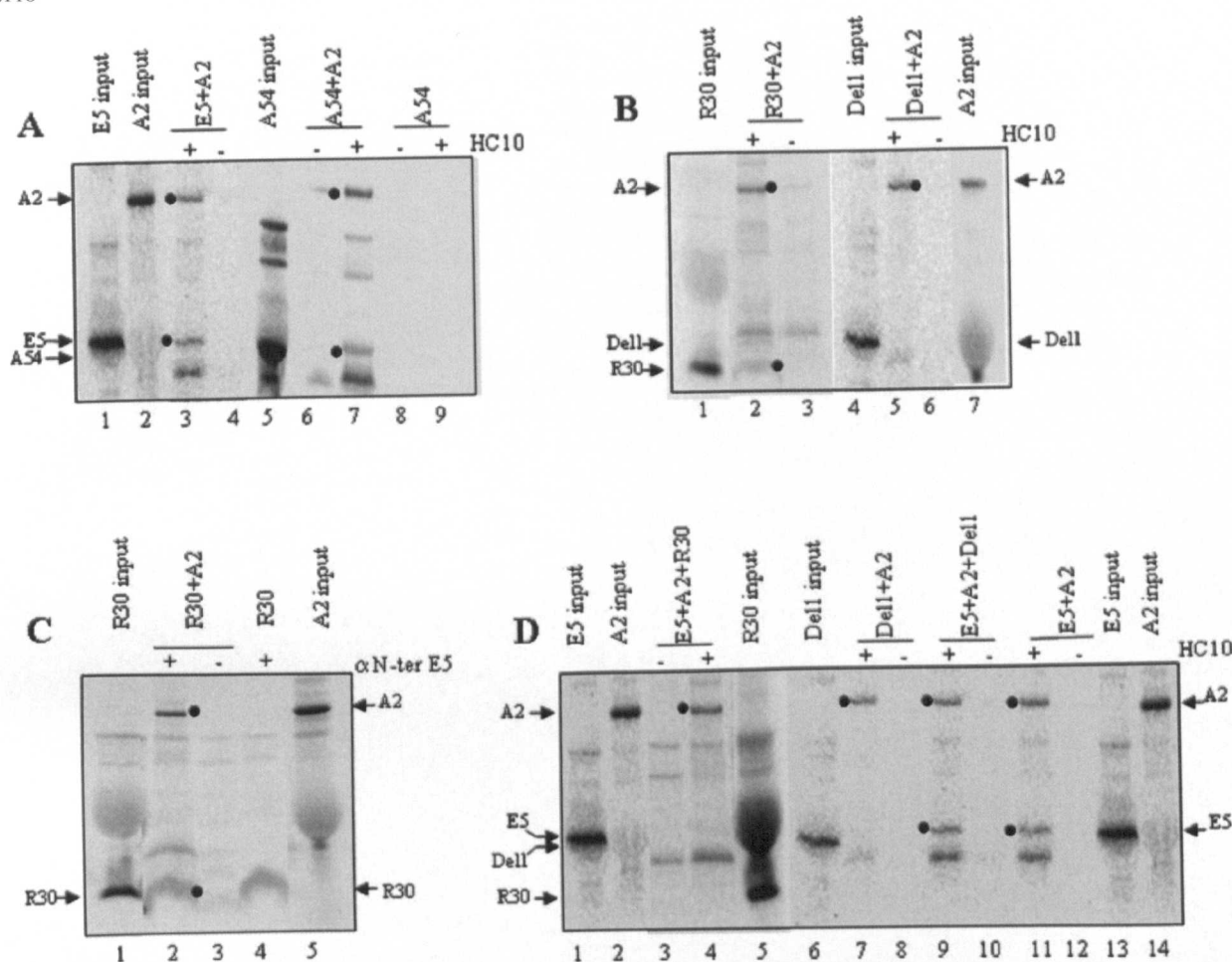


FIGURE 4 – Del1 does not bind HC. (a) E5 wt, A54 and A2 HC were transcribed/translated *in vitro* in the presence of ^{35}S -methionine and canine microsomes, mixed together and coprecipitated with mAb HC10 (+ lanes). Coprecipitates were observed only in the presence of antibody and of A2 HC. (b) R30, Del1 and A2 HC were processed as earlier. The only E5 mutant that did not interact with A2 was Del1 (lane 5). (c) R30 and HLA-A2 HC were transcribed/translated *in vitro* as above and coprecipitated with an antibody against the N-terminus of E5¹⁵ (+ lanes). Coprecipitates were observed only in the presence of antibody. (d) E5 wt and HLA-A2 HC were transcribed/translated and labelled *in vitro* as earlier; unlabelled R30 or Del1 were added to the reactions, and proteins precipitated with mAb HC10 (+ lanes). R30 competed with E5 wt and prevented its binding to HC (lane 4), while Del1 failed to do so (lane 9). Lanes 1 and 2 are the same as lanes 13 and 14, repeated for ease of comparison. In all panels, the coprecipitated E5, E5 mutants and HC bands are indicated by black dots; the other bands originate from the translated E5 protein (see input lanes) and may represent multimers of E5.

expressing E5 Del1, immunoprecipitation with mAb HA11 failed to precipitate HC (Fig. 1a, lanes 5 and 6), although in immunoblots HC was easily detected by mAb HC10 (Fig. 1a, lane 3) and E5 Del1 and E5 wt were expressed at comparable levels at both mRNA and protein levels (Figs. 1c (lanes 1 and 3) and 2c).

In *in vitro* coimmunoprecipitations, E5 wt and all the mutants containing the first helical domain (including R30, which contains only the first N-terminus 30 amino acids) coprecipitated with HLA-A2 HC (Figs. 4a (lane 7) and 4b (lane 2), shown only for A54 and R30). Despite the presence of nonspecific bands also in the lanes without antibody (some deriving from the translation of E5 and its mutants, possibly representing multimers of E5; see input lanes), mAb HC-10 coprecipitated in each case both A2 and the E5 proteins (marked with a black dot in Fig. 4). In contrast, Del1 failed to coprecipitate with HLA-A2 HC (Fig. 4b, lane 5). Coimmunoprecipitation between R30 and HLA-A2 HC was confirmed by using an antibody raised against the N-terminus of E5¹⁵. Also in this case, HLA-A2 HC coprecipitated with R30 (Fig. 4c, lane 2), validating the results obtained with mAb HA11 and HC10. These data indicate that the interaction between E5 and HC

takes place *via* the N-terminus first 30 amino acids. This was confirmed by competition experiments. The addition of unlabelled R30 (containing the first 30 amino acids) to the reaction prevented the interaction between E5 and A2 HC (Fig. 4d, lane 4), whereas the addition of unlabelled Del1 (amino acids 31–83) failed to compete with E5 and did not affect the E5-HC interaction (Fig. 4d, lane 9).

Taken together, these results conclusively show that E5 both physically interacts with HC and downregulates surface HLA I *via* its first hydrophobic domain.

Discussion

As is the case for the E5 oncoprotein of BPV,^{9–11,16} the E5 oncoprotein of HPV-16 prevents the transport of HLA I to the cell surface and retains the complex in the GA.⁴ We have argued that the Golgi retention of MHC I by BPV-4 E5 is due to at least 2 events: the impaired acidification of the GA and the physical interaction of E5 with the HC of MHC I.

HPV-16 E5 interacts with HC

The interaction between E5 and the MHC I HC is not confined to BPV E5. HPV-16 E5 forms a stable complex with the HC of the HLA-A3 (and/or A31 B60 B51) of HaCaT keratinocytes, and *in vitro* also with the HC of HLA-A1, -A2 and -B8. This interaction is therefore not specific for a particular HLA I haplotype/allele but it is likely to occur with most, if not all, HLA I. Complex formation between E5 and HC is responsible for downregulation of surface HLA I, as a mutant of E5, which fails to bind HC, also fails to prevent the transport of HLA I to the cell surface, as discussed later.

However, while the haplotype/allele of classical HLA I is not critical for E5-HC complex formation, E5 fails to downregulate nonclassical HLA I.⁴ These findings parallel those obtained with BPV-4 E5. BPV-4 E5 downregulates classical MHC I independently of its type¹⁶ and interacts with different HC,¹⁰ but fails to downregulate nonclassical MHC I and to interact with the HC of a nonclassical MHC I (our unpublished results). It remains to be seen if HPV-16 E5 is capable of binding the HC of nonclassical HLA I.

The first N-terminus hydrophobic domain of E5 is essential for downregulation of HLA I

HPV-16 E5 interacts also with 16 k subunit c, a component of the vacuolar H⁺-ATPase.¹⁷ Previously published deletion mutants of E5 have been proficiently used to map the domain of E5 mediating this interaction (see later⁸). These mutants and the new N-terminus deletion mutant of E5 showed the importance of the first N-terminus 30 amino acids in the interaction with, and downregulation of, HLA I. The absence of the middle and last hydrophobic domains does not prevent downregulation of surface HLA I, and the 30 amino acid-long N-terminus hydrophobic peptide is sufficient to prevent the traffic of HLA I to the cell surface. In contrast, the absence of the first hydrophobic N-terminus domain completely abolishes surface HLA I downregulation. The impaired function of this Del1 mutant is not due to diminished expression or inappropriate cellular localisation, as both expression and localisation are comparable to those of E5 wt or the other mutants. Furthermore, Del1 fails to interact with the HLA I HC both in HaCaT cells and *in vitro*, the latter observation confirming that the inability of Del1 to complex with HC is not due to improper expression or location. Of importance is the fact that R30, comprising only the first 30 amino acids, is capable of interacting with and downregulating HLA I to the same extent as E5 wt, thus largely eliminating the possibility that the failure of Del1 to downregulate HLA I is due to improper configuration, instability or any other feature which may be peculiar to this deletion mutant.

We have recently reported that BPV E5 interacts with the HC of bovine MHC I *via* its C-terminus domain.¹⁰ This contrasts with the results presented here with HPV-16 E5 but this difference is not surprising. Despite their differences in length and presumed conformation, BPV E5 and HPV E5 share numerous functional similarities, one of them being the interference with the proper function of 16 k ductin (and therefore of the vacuolar ATPase).⁷ BPV E5 and HPV-16 E5 interact with 16 k subunit c *via* different E5 domains: BPV E5 interacts *via* its 17th amino acid residue (Q in BPV-1 E5, N in BPV-4 E5^{18,19}), whereas HPV-16 E5 interacts *via* its second and third hydrophobic domains.^{8,20} Thus, the same effects, such as downregulation of MHC I and disablement of the proton pump, can be achieved by distinct physical interactions with HC and subunit c, respectively.

The interaction between HPV-16 E5 and 16 k subunit c has been deemed responsible for the impaired functioning of the pump and the consequent lack of acidification of the endomembrane compartments.^{21,22} Alkaline pH in the endolysosomes leads to delayed degradation and faster recycling of the epidermal growth factor receptor,^{22,23} thus promoting increased cell proliferation. Studies with deletion mutants, including those employed by us here, have mapped the 16 k-interacting domain of HPV-16 E5 to a relatively large region of E5 encompassing the second and third hydrophobic domains.^{8,20} The observation that HPV-16 E5 interacts with HC and 16 k *via* different domains suggests that E5 can in principle bind the 2 cellular partners at the same time, thus simultaneously promoting immunomodulation and cell transformation, partly explaining the pleiotropic effects of E5 expression.

We have commented before on the functional similarity between HPV-16 E5 and HIV-1 Nef. Both proteins prevent transport of classical HLA I to the cell surface in their natural host cells type,²⁴ bind to the HLA I HC *via* the same domains needed for HLA I downregulation²⁵ and neither downregulate nonclassical HLA I²⁶; both bind 16 k subunit c²⁷ and induce cytoskeletal rearrangements,^{28,29} and induce epidermal hyperplasia when expressed in the basal layer of the skin of transgenic mice.^{30,31} These similarities are intriguing and point to a process of 'convergent evolution' of these 2 proteins encoded by such different viruses.

Acknowledgements

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